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COLLOID CHEMISTRY OF THE PROTEINS

BY

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TRANSLATED BY

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SIR JOHN CASS TECHNICAL INSTITUTE, LONDON

PART I

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AUTHOR'S PREFACE

THE small volume of which Part I. is here presented has been developed from lectures delivered in the winter term of 1912–13. It by no means exhausts the materials of the colloid chemistry of the proteins even up to that date.

In response to many requests, it is proposed to collect in perspective the investigations carried out by the author and his co-workers in the course of many years, prominence being given to the most important results. Such fundamental work of other authors as is relevant has also been included in an attempt to round off the presentation, but for complete data the reader is referred to the well-known works of O. Cohnheim, R. H. A. Plimmer (translated into German by J. Matula), and T. B. Robertson.

The second part, to appear within a year, will include the relations of the proteins to neutral salts and to the salts of the heavy metals, to colloids and to ampholytes, the properties of the albumin gels, and, finally, the physical chemistry of the purest albumin so far prepared.

I take this opportunity of thanking most heartily Dr. Mona Adolf for her careful and critical reading of the proofs, and also my assistant and collaborator of many years, Dr. Joh. Matula, for drawing the figures.

WOLFGANG PAULI.

VIENNA.

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TRANSLATOR'S NOTE

The great importance of Professor Pauli's work on the proteins is ample justification for an English translation of this monograph, in which the work carried out by him and his collaborators is summarised and presented in relation to previous and concurrent research by other workers. By the application of the quantitative methods of physical and colloid chemistry a consistent theory of the behaviour of proteins, particularly in acid and alkaline solution, has been established.

In the translation a number of typographical errors have been corrected, a few slight alterations suggested by Professor Pauli have been made, and the indexes have been added. Otherwise no attempt has been made to do more than give the substance of the original in English.

The way of translators is hard : in this case it has been made much easier by the constant advice of Mr. Emil Hatschek, F.Inst.P., who has been good enough to read the whole of the translation in manuscript. For this great advantage I wish to record my gratitude to him.

P. C. L. THORNE.

Sir John Cass Technical Institute,
London.

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COLLOID CHEMISTRY OF THE PROTEINS

CHAPTER I

COLLOID CHEMISTRY AND THE GENERAL CHEMISTRY OF THE PROTEINS

It would be superfluous to discuss which of the constituents of the living cell are most important in vital processes. Proteins, lipoids, and certain inorganic salts are alike indispensable, and have a very intimate relation, both physical and chemical, one to another. There is, however, no doubt as to the central position of the proteins in the organisation of living matter. Apparently, they occur in nature in close connection with vital processes ; in the living cell they are completely irreplaceable ; and, above all, they alone display the specific properties of living matter. In consequence, the distinctions observed, not only between different kinds of organisms, but even between individuals of the same kind, reappear on chemical investigation as variations in the respective proteins.

Again, the proteins are capable of showing diversity and fine gradation, both in chemical structure and physical modification, to an extent which is lacking in any other class of substances. On account of the high molecular weight of even the simpler proteins, physical changes are brought about by minute quantities of substances of low molecular weight. Electrolytes in particular cause such changes very easily, owing to the amphoteric nature of the proteins.

The biologist attempts to arrive at a general expression for the profound and diverse phenomena of life, and finds that the rich variety of the reactions of the proteins confronts him as one of his greatest difficulties. Such progress as has been made has occurred mostly in recent years and has been largely due to the application of physical chemistry, and of the youngest branch

of that subject—colloid chemistry. The relations of this special branch of knowledge to the general chemistry of the proteins is so definite that a discussion of the position is desirable.

An independent branch of science arises when a number of observations are in strong contrast to previous records. The differences must be so striking that investigators are spurred on to an intensive study of the most glaring peculiarities, and the contrast between the new and the old knowledge serves to define sharply the bounds of the new discipline. As time goes on and the new knowledge increases, previous discoveries, which appeared to have little in common with it, are seen in a new light. Consequently, there is, in the first place, a period of detachment from the main stream of knowledge, followed by a second phase of reunion. When the new development is rich in observations and laws peculiar to itself, it will maintain a distinctive position even after the period of coalescence has begun, particularly when it contributes new methods of inquiry. In this way colloid chemistry has achieved its autonomous position.

Although there were distinguished workers in the field before him, Thomas Graham (1851) must be acclaimed as the father of colloid chemistry. Not only was he the first to set forth the most important properties of colloids, but he further emphasised the fundamental distinction between colloids and crystalloids—a distinction as wide as that between an organised substance and a lifeless mineral. Indeed, so vivid was this distinction to Graham and his immediate successors in research that any connection between the properties of crystalloids and colloids remained far in the background. Such a connection is, however, very plain in the considerations which formed the starting point of Graham's work. For he submitted the following line of thought: all substances show a greater or less difference in the rate or ease with which they pass from the solid or liquid state into the state of vapour; in many cases these differences are sufficiently pronounced to form the basis of a method of separation in fractional distillation. The differences in the rate of diffusion in a liquid are not less pronounced than in the rate

of dispersion into a gaseous phase. Is it not possible, therefore, to build up a process of separation and purification of dissolved substances on these differences? Graham's observation that substances which diffused with difficulty were more or less held back by parchment paper or by animal membranes indicated the method of purifying substances by *dialysis*, as he called the process. In this way salts and other substances which diffuse easily are removed without fundamental changes or chemical complications. It is well known how important dialysis has become, particularly in biochemical investigation. In Graham's hands it became the first general method of preparing pure *colloids*, a name which he gave to those substances which are distinguished by the following three criteria:—I. They diffuse very slowly or too slowly for the rate to be measured. II. They are unable to penetrate certain membranes, particularly some of animal origin. III. They lack the power to appear in the crystalline form. To these a fourth has since been added. G. Bredig, in particular, has shown that the work required to be done to separate the colloid from the solvent is very small or practically nil, as is shown by measurements on the evaporation or freezing out of the solvent. We shall see that all these criteria depend on one single property, which now appears to us as the significant characteristic of the colloid state.

The greatest progress which has been made since the time of Graham lies in the following straightening-out of ideas. Graham divided substances themselves into colloids and crystalloids. In which of these forms it appeared would thus seem to depend on the chemical nature of a substance. We now know, however, that the colloid or crystalloid state is no more than a physical manifestation of a dependent condition of matter. The same chemical substance can be obtained, according to circumstances, with colloid or crystalloid characteristics. This development is, in the first place, a result of improvements in methods of preparation. It is possible to obtain in the colloidal state all kinds of substances of low molecular weight, *e.g.*, metals, salts of alkalis and of the alkaline earths, etc. It has, moreover, been shown that for the production of the colloidal condition a state of fine subdivision is necessary, but not division into single

molecules. As all substances dissolve up to their solubility-value, that is, they become subdivided to molecular size, a colloidal dispersion only becomes possible when the solubility value is exceeded. So that difficultly soluble substances lend themselves most readily for transformation into the colloidal state (*e.g.*, metals, many metallic sulphides and hydroxides, fats and resins, with water as dispersion medium). If, on the other hand, it is desired to obtain salts or other substances soluble in water in the colloidal state, methyl alcohol, acetone, etc., must be used as dispersion medium. Of course, it is also essential that the division of the particles must be sufficiently fine for the particles to remain free swimming and evenly distributed throughout the bulk of the liquid ; otherwise we are dealing with a slime, a coarse suspension, or an emulsion.

Such fine dispersions, or, as we can now call them, *colloidal solutions*, pass easily through a filter. Their real nature and the difference between them and true molecular solutions was first made clear by the use of the ultramicroscope of Zsigmondy and Siedentopf, which made the degree of dispersion optically apparent and permitted a closer determination of the size of the particles. According to R. Zsigmondy, we can distinguish by their mean linear dimensions coarse suspensions with particles of $0{\cdot}1\mu$ diameter, and colloidal solutions with particles of $0{\cdot}1\mu\mu$ diameter. The latter pass without a break into the various molecular solutions as the size of particle decreases.

Wolfgang Ostwald is the author of the very suitable terms *dispersion medium* (continuous phase) and *disperse phase* for the component parts of a colloidal system. The degree of dispersion (dispersity) is the quotient of the total surface and the total volume of the dispersed particles.

It is easy to see that there are fundamentally two general methods of preparing a colloidal solution of a substance : (1) *dispersion methods*, in which large particles or aggregates are broken up, as in electrical dispersion, partial solution of coarse precipitates or peptisation ; and (2) *condensation methods*, in which a molecular solution is caused to become super-saturated, either by sufficient dilution or the addition of certain substances

until formation of a finely divided disperse phase occurs. The theory of the main methods of formation of the colloidal state of all substances and the general formulation of the conditions of their existence has been developed by P. P. von Weimarn.

Finally, solutions of colloidal character are obtained when substances of very *high molecular weight* are dissolved in water. The tendency of the large molecules to associate and polymerise can still further increase the colloidal properties of these solutions. Albumin, starch, glycogen, etc., are included in this group of substances, whose main characteristic is that they occur exclusively in the colloidal condition.

Let us suppose that, instead of a dilute solution of a substance of low molecular weight, the same total quantity of material is present as much larger particles, which, in consequence, are fewer in number. Clearly such properties as depend on the number and size of the particles will in consequence be considerably altered. The osmotic pressure would fall to a small value; the power of penetrating membranes, as also the rate of diffusion in general, would become minute; while the directive force which orientates particles for formation of crystals becomes very weak or can be no longer developed. Accordingly, no qualitative difference between typical dilute solutions, as of sugar or salts, and colloidal solutions is to be expected, but merely a gradual transition from the one to the other.

In the second place, the characteristics of the latter solutions would appear. First, whereas in a very dilute solution of a substance of low molecular weight, the volume of the solid can, as a rule, be neglected, in a colloidal solution the volume of the particles is appreciable in consequence of their size. Secondly, the particles are often so large as to be visible when refined optical methods are used, *e.g.*, by observing with the naked eye at right angles the fog produced by a bright beam of incident light (*Tyndall's phenomenon*), or the diffraction images in the microscope (ultramicroscope). The disperse phase develops physical surfaces in the medium of dispersion, so that the most varied reactions of the two phases can be fixed or modified according to the physical properties of these surfaces.

The work, in particular, of J. Perrin and of The. Svedberg * on colloids has demonstrated that in microscopically visible systems the properties of fine dispersions can, in certain directions, be brought into quantitative relation to those of typical solutions. We must here consider in rather more detail the work of Perrin, the upshot of which was that it is possible directly to ascertain the gas constant, and, above all, Avogadro's number for dispersions (as, for instance, of gamboge or mastic) with astounding accuracy.

The law of Boyle and Gay Lussac—$pv = RT$—was expanded by Avogadro into his incredibly fertile hypothesis that for the same conditions of pressure and temperature the same volume of any given gas contains the same number of molecules. This development depended on the discovery that the weights of equal volumes of two gases under the same temperature and pressure conditions are proportional to the respective molecular weights. Therefore the gram molecule (the molecular weight in grams) of different gases contains the same number of molecules.

Van t'Hoff † has shown that the same law holds for dilute solutions, provided the osmotic pressure of the solution against the pure solvent is substituted for the gas pressure. The constant R has the same value as for gases, and the solution of gram molecules of different substances (provided no association or polymerisation occurs) gives here once again the rule of Avogadro that in each solution the same number of molecules is present. This number is a well-known constant, and will be indicated as Avogadro's number N.

The kinetic theory of gases, largely the work of Clausius and Maxwell, has for its fundamental assumption the idea of movement of the molecules. These movements increase with a rise in temperature, and cease altogether at absolute zero. Avogadro's hypothesis can be deduced from the gas laws by

* "Die Existenz der Moleküle." Leipzig. 1912.

† It is extraordinary that the fact that Graham used the analogy between solutions and gases as the basis of his work has not been emphasised. Of course, he only used the comparison for a special case, and it was left to Van t'Hoff to develop it in a general form and apply it quantitatively to dilute solutions.

applying the laws of mechanics. For a gram molecule of a gas (or dissolved substance) the simple formula of the kinetic theory reads :

$$pv = \frac{2}{3} Nw = RT,$$

where N is Avogadro's number and w the mean kinetic energy of one molecule of gas or solute.

The kinetic theory of gases has, moreover, caused the behaviour of gases under high pressure to be compared with that of liquids, with the result that the apparent difference in molecular condition in the two cases is not found to exist.*

J. Perrin † has applied the conclusions of Van t'Hoff for solutions to the cases both of pure liquids and of suspensions. To quote his own clear summary :—" Van t'Hoff's law states that a molecule of ethyl alcohol has the same energy in an aqueous solution as when it is vaporised therefrom. It would also possess the same energy in solution in chloroform, that is, when surrounded by chloroform molecules, and similarly in solution in methyl or propyl alcohol. It may therefore be supposed that its energy is also the same in ethyl alcohol solution ; in other words, when it is a constituent molecule of pure ethyl alcohol. So that, by admitting that the molecular energy is the same in the liquid as in the gaseous state, we can now state that at the same temperature the mean kinetic energy of all molecules of every liquid is the same, and is proportional to the absolute temperature.

" The general hypothesis can, however, be still further developed. From what has been stated above, the heavy sugar molecules in a sugar solution have the same mean energy as the mobile molecules of water. Now the sugar molecule contains 45 atoms ; that of quinine sulphate more than 100. One can enumerate still heavier and more complex molecules, to all of which the law of Van t'Hoff is applicable, for no limit is placed to the size of the molecule. Consider, now, a particle made up of several molecules, say a dust particle. Is it likely it will conform to a different law under the bombardment of the

* See W. Nernst, " Theor. Chemie.," 1907, p. 226.

† See *Koll. Chem. Beihefte*, 1910, **1**, 221, for complete literature.

surrounding molecules ? Will it not rather behave like a large molecule, so that its mean energy is that of a single molecule ? "

The movement of suspended particles was first described by the botanist Brown in 1827. Various later workers (Wiener, M. Gouy) confirmed its existence, and traced its origin to the movements of the molecules. Perrin was able to produce gamboge suspensions of particles of the same size by fractional centrifuging, and, by counting the number of particles at various depths in a cell under the microscope, was able to show that the distribution under the force of gravity followed the same exponential law as is found, for instance, in the distribution of the air in the atmosphere. Thus a difference of 10μ in depth in the cell gave the same decrease in number of particles as occurs in a height of 6 kilometres in the atmosphere. The value of the constant N, calculated from the distribution equation of various emulsions is 70×10^{22}, a value for Avogadro's constant which agrees exceedingly well with that obtained by Van der Waals.

From the above and from other demonstrations of the applicability of the kinetic theory of gases to colloid systems (*see* The. Svedberg), we can perceive no fundamental distinction between the behaviour of particles of molecular dimensions and that of colloid particles up to those of a coarse suspension. We shall see further that a similar transition occurs in electrochemical properties.

Under these circumstances, there can be no question of a sharp demarcation between the general chemistry of substances of high molecular weight, such as proteins, and their colloid chemistry. The difference is certainly not a matter of size of particles, but rather a matter of structure. If a metal or a simple compound is brought to the colloid state, the dispersed particles are always aggregates of simpler but similar smaller portions. If, on the other hand, the particles are those of a non-associated substance of high molecular weight, of the same degree of dispersity as the above, we have particles formed of a combination of several heterogeneous groups of atoms (for instance, albumin, which is formed of various amino-acids). We have so far absolutely no knowledge of the distribution of

these groups on the surface or inside the molecule. At first sight it appears that the second example is more complex than the case of the colloidal metal ; but one must not overlook the fact that the various groups which build up the complex compound often retain their characteristic properties, and stamp the whole molecule with their physico-chemical behaviour. These properties of compounds of high molecular weight can therefore, in favourable circumstances, throw light on many general colloidal phenomena, as happens in many respects with the proteins. Although we regard the colloid chemistry and the physical chemistry of the proteins as inseparable, we must at the same time emphasise every tendency to a relation between their colloid chemistry and their structure. This connection was the object of our own researches, and is one which will undoubtedly be largely developed in the near future.

CHAPTER II

CONDITIONS OF STABILITY IN PROTEIN SOLUTIONS

THE definition of a colloid system as a dispersion within certain limits of subdivision is satisfactory in emphasising a general property ; it does not, however, suffice to define special cases when certain hypotheses become necessary to complete the description. These serve to subdivide colloids into broad classes. Thus Wo. Ostwald has devised a classification based on the state of aggregation of the disperse phase. Colloids with solid disperse phase are *suspensoids,* those with liquid particles *emulsoids.* If the dispersion in such systems is reduced so that the disperse phase coalesces into macroscopic masses, the suspensoids give solid coagula, which settle out of the liquid. The emulsoid particles, in contrast, flow together into drops, which frequently unite to form a separate liquid layer.

"If a 10 per cent. gelatin solution is precipitated at 30° by addition of a neutral salt (*e.g.*, sodium sulphate) and allowed to stand for some hours at the same temperature, the gelatin is found in both layers, the upper more liquid one containing but little gelatin, the lower layer being rich in gelatin (Wo. Pauli and P. Rona *). A similar effect has been observed and further studied by K. Spiro † when casein is coagulated by heat."

The classification of colloids from another point of view is important in characterising the physical properties of the proteins. Both in the case of typical solutions and in that of colloidal solutions, a greater or less combination between the particles and the solvent or continuous phase has been shown to exist. Crystalloids are said to be *hydrated* (or, in general, *solvated*) in solution when water is attached to the dissolved

* *Beitr. z. chem. Physiol. u. Path.*, 1902, **2**, 1.
† *Beitr. z. chem. Physiol. u. Path.*, 1904, **4**, 300.

molecules in a stoichiometric proportion. In some instances this hydration is sharply defined (as for sulphuric acid and ferric chloride), but in other cases the hydration is variable and shows a continuous alteration with dilution and with change of temperature. We can describe such phenomena with more accuracy as addition of water (or formation of envelopes) by the particles, rather than as combinations in the more restricted sense of the word.

Such an addition of the medium occurs on the particles of many colloids, and is described as *hydration* in the wider sense, and as *imbibition* when the adding of water occurs together with penetration of water into a large aggregate. The indications of hydration of a disperse phase are a disproportionately great viscosity of the solution, the decreased activity of the movement of the particles as displayed in diffusion or in an electric field, and an alteration in volume or density in the direction of compression of the medium. The latter is made obvious by a comparison of the volume with that calculated from the volume of the particles plus that of the medium, or by the increase in the effective concentration of a substance in solution, as also by anomalous behaviour when the solvent is removed by freezing or evaporation, owing to a stronger partial combination. In some cases there is an evolution of heat on solution in the medium.

We shall see that hydration of particles of albumin solutions has been demonstrated, and, consequently, dissolved proteins are classed among hydrated (solvated) colloids. These are called hydrophile by J. Perrin, and in general *lyophile* by H. Freundlich. Colloids with non-solvated particles are named hydrophobe (*lyophobe*), and although the definition of suspensoids and emulsoids is on a different basis, in practice the two classifications approximately coincide. The embarrassing use of the double classification according to the state of aggregation or solvation of the disperse phase is strongly to be deprecated. With water as continuous phase the obvious notation *hydro-* and *anhydro-colloid* is useful. Hydrated colloids are usually more stable in solution than anhydro-colloids.

The *rôle* of solvation in the stability of various colloid

systems is brought out most clearly when the effect of the *electric charge of the particles* on the equilibrium of the system is investigated at the same time. When the behaviour of colloids in an electric field is explored, a movement of the particles towards one of the poles can ordinarily be demonstrated. If electro-negative, they wander to the anode (*e.g.*, metals, metallic sulphides, mastic suspensions, silicic acid, etc.), or, if positive, to the cathode (*e.g.*, metallic hydroxides). The work of H. Schultze, H. Picton and S. E. Linder, W. B. Hardy, W. Biltz, J. Billiter, H. Freundlich, and others has shown that the *electric charge on the particles of these colloids is essential for their stability in solution.* The removal of the charge on the particles leads to a cessation of movement in an electric field, and also to precipitation. This fact was first noted and precisely stated by W. B. Hardy. Later researches show that even an incomplete discharge of the particles (for oil-emulsions $\pm$ 0·03 volt) leads to coagulation (R. Ellis * and F. Powis †).

This discharge and precipitation of the colloidal solution can be brought about in various ways :—

I. By addition of electrolytes, in which case relatively low concentrations of the ions are sufficient. Positive ions are effective in precipitating negative sols and negative ions for positive sols (*Hardy's Rule*). The potency of the ion increases disproportionately with its valency, polyvalent being more effective than monovalent, and of the latter H- and OH-ions are the most powerful (H. Schultze, W. B. Hardy, H. Freundlich).

II. Oppositely charged colloids neutralise each other, and are mutually precipitated. Excess of one of them makes precipitation incomplete or may prevent it altogether (W. Biltz, J. Billiter).

III. If secondary reactions are neglected, precipitation occurs on the oppositely charged pole when a colloid is subjected to an electric field.

IV. The charge on the particles is decreased, even to the

* *Zeitsch. physikal. Chem.*, 1914, **89**, 145.
† *Zeitsch. physikal. Chem.*, 1914, **89**, 186.

point of coagulation, when the colloid is subjected to electrical radiations. For instance, as can be foreseen, the β-rays of radium precipitate a positive colloid, *e.g.*, ferric hydroxide (W. B. Hardy, V. Henry and A. Mayer, A. Fernau and Wo. Pauli *).

Let us next consider the connection between these phenomena and those displayed by a typical true solution. Here also is a stability dependent on charged particles, the ions. We know no means of causing a notable separation of the ions from solutions; but neutral particles can be deposited from the solution in the form of a precipitate. The property discussed above gives a suitable analogy to the behaviour of normal electrolytes, if we so choose the latter that the limit of solubility of the neutral particles formed is exceeded. The precipitation of barium as sulphate, the electrolytic deposition of a metal from its salt, and similar reactions can be expressed in a form analogous to the precipitation of colloids.

We can state, in general, that whenever the neutral particles of a colloid are not stable in solution it is precipitated by removal of the electric charge.

There are, however, colloids in which the neutral particles are stable, and thus electrical neutralisation does not lead to precipitation. This observation was first made with natural albumin (Wo. Pauli), and it holds also for starch, gelatin, agar, gum arabic, some forms of silicic acid, etc.

The *first group* of colloids, with *neutral particles, which are unstable in solution*, includes the hydro- (or lyo-) phobe, or anhydro-colloids (the suspensoids of Wo. Ostwald and P. P. von Weimarn), while the *second group*, with *stable neutral particles*, includes without exception hydrated or solvated colloids.

We must now consider a more important factor. For a colloid to be stable in an electrically neutral state, it is necessary for the discharged particles formed to be sufficiently hydrated. As we shall see, many proteins (casein, globulin, boiled albumin, etc.) form heavily hydrated particles in solution when electri-

* *Biochem. Zeitsch.*, 1915, **70**, 426; *Kolloid Zeitsch.*, 1917, **20**, 20, for the literature.

cally charged, but precipitate in the electrically neutral state, as the particles lose their water of hydration when discharged.

The importance of solvation and electric charge of the particles in determining the stability of colloids is shown by numerous researches. The questions of the origin of the electric charge and of the mechanism of discharge and precipitation of the colloid particles lead us, on the other hand, into a field of various conflicting theoretical speculations.

It would appear impossible to suggest a single source of the electric charge which would be applicable to all colloid systems. But in the case of the proteins, as we shall show, all experience points to the conclusion that those which behave as electrolytes owe their charge to typical ionisation processes. Similarly, silicic acid, stannic acid, tungstic acid, etc., which give positive H-ions, form particles which are charged negatively, as also do colloidal solutions of the noble metals, when positive metal ions are produced. Colloidal metallic hydroxides, on the other hand, can be regarded as complex salts of the metallic oxide with the salt from which they are prepared by hydrolysis and dialysis (see the recent work of Wo. Pauli and J. Matula *). These complex salts consist of a positive colloidal ion and a simple anion. For example, a ferric hydroxide sol prepared from ferric chloride contains the ions $x\mathrm{Fe(OH)_3} \,.\, y\mathrm{Fe}^{\cdots}$ and $3y\mathrm{Cl}'$.

Difficulties crop up when this purely ionic conception is applied to colloids in which at present no notable ionisation has been detected: for example, emulsion of resins, and such like bodies. But even here the ionisation resulting from slight saponification at the surface of the particles is not excluded. The theory of the charging of colloid particles as an ionic process in which one ion is very large has been developed by J. Billiter. The particles have the characteristics of an ion with the charge of the electrode which repels them.

This view of the origin of the charge on the particles of colloids leads to a theory of the discharging processes which is most simply regarded as an ionic reaction at the surface of

* *Kolloid Zeitsch.*, 1917, **21**, 49 (literature).

the disperse phase. This conception is quantitatively worked out by H. Freundlich in his theory of adsorption of ions.

When a complex salt is dealt with (as, for example, most metallic hydroxide sols), two kinds of electric neutralisation by electrolytes take place: (1) suppression of ionisation and reduction in solubility due to a considerable quantity of added ions, and (2) low solubility product of one ion, which precipitates in very low concentrations. The action of chlorides, nitrates, etc., on ferric hydroxide sol or ceric hydroxide sol is an example of the first, while that of oxalates, tartrates, sulphates, etc., is an example of the second method of neutralisation.* Analogous behaviour is found with proteins.

Bredig's theory of the process of precipitation is most widely known. According to this view, neutralisation removes the electrostatic repulsion between the particles, and owing to the tendency of surface energy to sink to a minimum, a reduction in surface occurs by the particles joining together. It cannot be doubted that this is an important factor in the process of precipitation. In particular, the fact that there is a power of association between the neutral particles in a molecular solution must not be overlooked. It comes into play in the formation of precipitates and in crystallisation, and hence can scarcely be neglected in the case of colloids. But our knowledge in this direction is very slightly developed, and any precise connection between surface tension and molecular association is lacking, though both depend on chemical constitution. Developments in this border-line field are likely to come in large part from the study of colloid chemistry.

Zsigmondy, again, considers the question of whether a dried colloid is again dispersed by mere addition of the dispersion medium as a basis of classification. Colloids which redisperse he calls *resoluble*; those that do not, *irresoluble*. This criterion is also useful up to a point in facilitating the general comprehension of colloidal behaviour. The extreme lyophobe colloids are irresoluble, while typical hydro-colloids are resoluble. The redispersion of a dried colloid is conditioned by the ionising power of the continuous medium, and the tendency of the

* See Wo. Pauli and J. Matula, *loc. cit.*

colloid to form ions, as well as by the solvation and aggregating power of the disperse particles due to their varied molecular association and surface tension.*

Having cleared the way by this discussion, we will now briefly collect the facts which bear on the stability of proteins.

Some proteins are hydrophobe, some have hydrated particles; and the conditions under which they are stable are different in the two cases. Proteins of the first group exhibit Hardy's law of instability at the neutral point when treated with electrolytes. To this group belong heat-coagulated albumin, casein, salt-free globulin, acid-albumin, etc. On the other hand, glutin, natural albumin, and the other proteins of the second group are stable in solution, even when the particles are not electrically charged. The electric charge is, however, quite an important factor in the stability of the lyophile or hydrated proteins, for it is an accepted fact that only neutral particles are precipitated from solution by electrolytes. Accordingly, all means of displacing albumins from solution will show an optimum at the point of electric neutrality, a condition which will be dealt with in detail later on. Generally, the difference between lyophobe and lyophile albumins lies in the fact that in the first case mere discharge of the particles (*e.g.*, by an electric field) leads to precipitation, while in the second case discharge must be combined with effects which lead to an alteration in the relation of the particles to the solvent, so that their lyophile character is reduced or lost altogether. Saturation of the solution with certain alkali salts, the addition of alcohol or phenols, etc., brings about the latter conditions.

Such variations in stability as are indicated finally by a visible coagulation form yet another class of changes to which colloids are prone. There is no fixed nomenclature for these changes, which lie on the border line of the physical and the chemical, and they are termed in general the alteration of state of colloids.

Such changes are classed as *reversible* or *irreversible* coagula-

* Some irresoluble colloids, after precipitation by excessive dialysis, can be redispersed by addition of some electrolyte. This effect, often called *peptisation*, really depends on the formation of complex salts, as Zsigmondy has shown in the case of stannic acid gel.

tions. The process is termed reversible when a simple reversal of the procedure which led to coagulation causes redispersion. For instance, a jelly which has set on cooling melts again when heat is applied ; whereas a protein which has been coagulated by heating does not redissolve on cooling, and is therefore said to have undergone an irreversible coagulation.

If, when we cause a coagulation to pass again into the suspension which originally existed, we proceed in this reversal by the same path as that traversed in the coagulation process, only in the opposite direction, so that the same state is produced by the procedure, independently of the order of the procedure, such a process is called a *homodrome reversible* change. If, however,

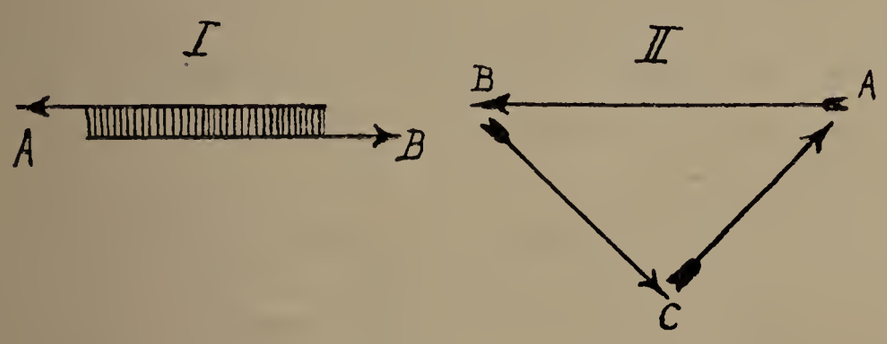


FIG. 1.—Homodrome and heterodrome changes of state.

the reversal must go by a path different from the original one, so that similar procedure gives rise to various states of existence in the colloid, the process is *heterodrome reversible*. Homodrome reversibility is only realised in practice in a very restricted range and in a gradual course of reversion of a colloid.*

Another property of colloids which was noticed even by Graham is connected with their reversible coagulation ; that is, their tendency to alter with age, which gives them the qualities of a more or less unfinished product. The reactions and alterations incidental to the preparation of a colloid do not, in general, terminate with the appearance of the colloidal character, but gradually proceed further after this point is reached. Lyophobe colloids, in particular, show these changes,

* Homodrome reversible changes, in the strict sense, do not occur in nature, but merely form an ideal case. Nevertheless, the above classification has its practical uses. See Wo. Pauli, *Naturw. Rdsch.*, 1902, **17**, Nos. 25, 26, 27.

mostly in an alteration from a state of high dispersion to one of lesser subdivision and of slight stability which in the end brings the colloid or crystalloid impurities in the solution into activity, so that part of the disperse phase is precipitated. This effect is known as the *ageing* of the colloid; and the fact that the response of the colloid to any action often continues to appear for some time after the action has ceased, the *hysteresis* of the colloid. This effect occurs equally with a stabilising or destabilising influence.

Sols of metallic hydroxides, such as ferric hydroxide sol or ceric hydroxide sol, show on ageing a continuous decrease in viscosity and an increase in electrical conductivity.* As no change in the concentration of electrolytes can be demonstrated, a gradual decrease in the hydration of the particles must be assumed to occur with lapse of time. This effect is also noticed in another research on hydroxide gels.†

Ageing effects are very important when dealing with proteins. Serum, which has been freed from insoluble globulins by prolonged dialysis and subsequent filtration, must be allowed to stand for months until the further precipitation of globulin, which invariably occurs, is complete, and the serum is quite clear. The effect of storage and the changes in activity which depend on it are particularly important in the colloidal toxins and antitoxins.

Graham used the term *sol* for a colloid system in the liquid state, while the name *gel* is applied when a partial or complete separation of the disperse phase has occurred, or when the colloid has set as a whole to a jelly. The term gel is, however, very difficult to define accurately, and is used in many different senses in the literature. A closer consideration of the gels, which we would differentiate as systems with a structure from the jellies, in which a demonstrable structure is not assumed and which correspond to the sols (Lottermoser), will follow later.

In the following chapters we shall again make use of the views on the classification and alteration of state of colloids which we have explained above.

* A. Fernau and Wo. Pauli, *Kolloid Zeitsch.*, 1917, **20**, 20.
† Wo. Pauli and J. Matula, *Kolloid Zeitsch.*, 1917, **21**, 49.

CHAPTER III

THE ELECTRIC CHARGE ON NATURAL SOLUBLE ALBUMIN: THE ISO-ELECTRIC REACTION

While the general experience of the behaviour of lyophobe colloids paves the way to a realisation of the connection between the electric charge and the stability of the disperse phase, it is obviously necessary to obtain definite proof that albumins behave in a similar way.

A direct method of showing the charge on protein particles is that of investigating the direction of their motion in an electric

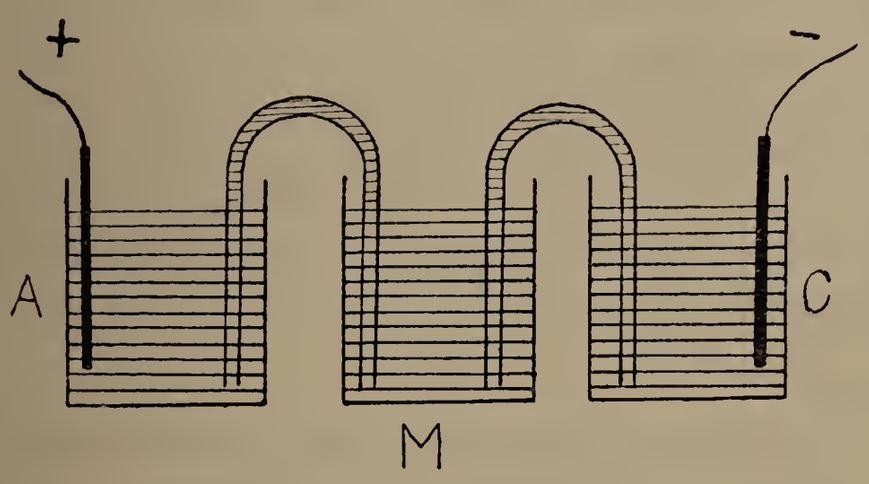


Fig. 2.—Electrophoresis of albumin.

field (*electrophoresis*). This investigation was first undertaken to find out the effect of the addition of various substances to the protein solution. A simple transport apparatus in which that portion of the colloid solution which is in the neighbourhood of the poles can be abstracted for analysis serves the purpose. Thus Wo. Pauli,* in the original investigation of natural proteins, used a series of three small beakers connected by syphons filled with water. Platinum electrodes were put into the two outer beakers, and after the current had been passed for some time the syphons were withdrawn and the protein content of the

* *Beitr. z. Chem. Physiol. u. Path.*, 1906, **7**, 531.

beakers determined (*e.g.*, by a nitrogen estimation by Kjeldahl's method).

In this way it was shown that serum albumin, after the removal of electrolytes by dialysis for some weeks, tends to move but slightly in an electric field. This fact was only obvious on prolonged electrophoresis. The addition of alkali or of alkaline salts causes a marked wandering to the positive pole, while added acid or acid salts promote a movement towards the negative pole. Neutral salts of the alkali or alkaline earth metals do not have a noticeable effect on the direction of movement of the protein. When, however, electrolysis has produced acid at the anode and alkali at the cathode, these secondary products cause the albumin to become charged, so that positive albumin is produced in the acid, and negative albumin in the alkali. In consequence the protein is repelled from the corresponding pole and forced towards the centre. It is therefore necessary, in using the above apparatus, to control this electrical repulsion by determining the content of the central beaker. In the following tables several examples of results are given. The albumin content is measured by the volume of N/4 acid required to neutralise the ammonia formed in a Kjeldahl determination. (A = anode cell; M = middle cell; C = cathode cell.)

Table 1.

Horse-serum dialysed for nearly Seven Weeks.
250 volts and 2×10^{-5} amps.

(*At the end of the experiment there was no appreciable alteration in reaction in the three vessels.*)

Time of Electrophoresis.											
3 hours.			6 hours.			24 hours.			48 hours.		
A.	M.	C.	A.	M.	C.	A.	M.	C.	A.	M.	C.
3·7	3·8	3·85	4·0	3·85	3·95	4·15	4·05	3·8	4·03	4·8	3·9
3·85	3·75	3·75	4·0	3·95	4·0	4·55	4·1	3·8	4·03	4·8	3·9
Indifferent.			Indifferent.			To the anode.			To the anode, with repulsion towards the central cell.		

Table 2.

Effect of Addition of Acids and Bases.

(*Time—6 hours in all cases.*)

Horse-serum. + 0·01N acetic acid.			Ox-serum. + 0·01N hydrochloric acid.			Horse-serum. + 0·01N sodium hydroxide.		
A.	M.	C.	A.	M.	C.	A.	M.	C.
7·05	7·55	8·20	7·4	8·05	9·1	8·3	7·65	6·8
7·00	7·45	8·25	7·45	8·05	9·05	8·3	7·60	6·8
→			→			←		
To the cathode.			To the cathode.			To the anode.		

Table 3.

Effect of Addition of Salts.

(*Time—6 hours.*)

Horse-serum. + 0·01N NaCl.			Horse-serum. + 0·01N $CaCl_2$.			Ox-serum. + 0·01N $NaHCO_3$.			Ox-serum. + 0·01N NaH_2PO_4.		
A.	M.	C.	A.	M.	C.	A.	M.	C.	A.	M.	C.
7·6	7·9	7·55	7·4	7·5	7·4	10·15	8·25	6·25	7·95	7·85	8·05
7·5	7·9	7·55	7·4	7·6	7·5	10·00	8·25	6·2	7·8	7·85	8·05
Indifferent, some repulsion.			Indifferent, slight repulsion.			To the anode.			To the cathode.		

The investigation was made more sensitive later on by the construction of a more perfect apparatus * for detecting movement in the electric field. We will now describe this apparatus, as it is capable of wide application.

A large U-tube is provided with a tap in the middle of each limb; and near the top of each limb two other U-tubes, about half as big as the central one, are fused on as shown. These two tubes are joined at their lowest points by a tube also provided with a tap. The large U-tube can be fitted from the bottom at C. It is charged in this way with the colloid under examination

* C. Landsteiner and Wo. Pauli, "25 Kongr. f. Innere Medizin, 1908."

until it is full to a level just above the two open taps. These are then closed, avoiding the inclusion of air bubbles. The rest of the apparatus, after cleaning, is then filled with a liquid of the same conductivity, *e.g.*, a solution of KCl or similar salt which is in equilibrium with the colloidal solution. This liquid is levelled by opening tap III., which is then closed, and I. and II. opened. The liquid lying above the colloid must be less dense than the colloid, and a clean line of division is thus obtained. When the current is switched on, negative colloids rise up through I., positive colloids through II. With a neutral sol no movement on either side occurs. At the end of the electrophoresis taps I. and II. are closed and the contents of the parts of the U-tube above the taps removed by a fine pipette and tested. The apparatus can also be used as a null instrument, for instance, to find the point when no migration of the albumin occurs, for which purpose filling with trichloracetic acid gives a high degree of sensitiveness (Wo. Pauli and Samec). L. Michaelis introduces crystals of sulphosalicylic acid for the same purpose. This apparatus can further be used for the direct measurement of the rate of motion of charged colloid particles in the electric field, provided the distance which the boundary line moves in a given time is determined with a known potential gradient.

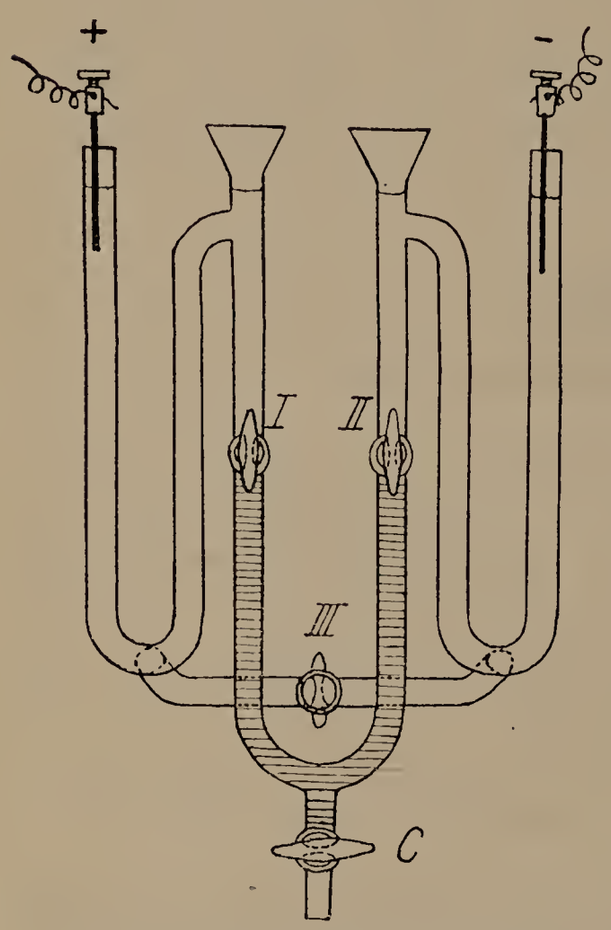


FIG. 3.—Improved electrophoresis apparatus.

By the use of this apparatus, it can be shown that proteins, such as albumin and glutin, regularly show an electro-negative

charge when in the natural condition (Landsteiner and Pauli). The same fact was noted a little later and independently by F. Botazzi * and by L. Michaelis.† The latter used Landsteiner and Pauli's apparatus, and, by employing non-polarisable electrodes, was able to work with colloids to which greater quantities of electrolytes had been added. An anode of silver in potassium chloride, and a cathode of silver in silver nitrate, or of copper in copper sulphate, or similar combinations, are useful for this purpose. It is always important to avoid potential differences at the junctions of the separating liquids by the use of solutions of as nearly as possible the same conductivity. The ions of the electrolytes should not differ greatly in mobility. In the apparatus described above, the short electrodes are situated so far from the surface of the colloidal solution that with 0·01 N solutions no disturbances due to electrolysis occur if electrodes of platinum wire are used and the application of the current is not too prolonged.

As at first albumin particles move slightly to the anode owing to their negative charge, and on addition of increasing quantities of acid become positively charged, it is clear that at a certain acid concentration an indifferent point in the sign of the charge will occur. Under these circumstances albumin shows no electrophoresis in either direction. Hardy called a colloid which showed no potential difference between the particles and the medium an *iso-electric* colloid. The application of the iso-electric reaction of proteins as an important method of differentiating between them has been emphasised in many cases by L. Michaelis, who has also devoted much time with most fruitful results to the theory and practical determination of the *iso-electric point.*

The variation in electrical properties of the proteins on addition of acid or alkali is connected with the property they display of behaving as bases towards acids, and as acids towards bases. This property has been well known for a long time, and substances which combined the properties of a weak acid with those of a weak base were called *amphoteric electrolytes* by

* *Atti. R. Accad. Lincei*, 1908, **17**, 49–57.
† *Biochem. Zeitsch.*, 1908, **16**, 81.

Bredig. The amphoteric electrolytes are generally asymmetrical with respect to their power of dissociating into $H^{\cdot}$ or OH' ions, for either the acidity or the basicity is predominant. Only water, which dissociates $H_2O \rightleftharpoons H^{\cdot} + OH'$ is symmetrically amphoteric.

The application of the law of mass action to amphoteric electrolytes by G. Bredig, J. Walker and H. Lundén * has resulted in the development of a theory applicable to them which has proved particularly fruitful in dealing with the proteins. We will next give some applications of the mass action law to electrolytes, which we shall have to use repeatedly.

The dissociation of an acid, *e.g.*, the weak and slightly ionised acetic acid $CH_3 . COOH \rightleftharpoons H^{\cdot} + CH_3 . COO'$ is expressed by the equation $C_{H^{\cdot}} \times C_{CH_3COO'} = K \times C_{CH_3COOH}$, where C is the molecular concentration of the various substances, and K is the dissociation constant of acetic acid. The value of K for a weak electrolyte is largely independent of dilution (W. Ostwald). This equation holds for every solution, provided the proper molecular values are inserted, in this case those for the acetation, hydrion and undissociated acetic acid. If the product on the left hand of the equation is increased by a rise in the concentration of acetations or hydrions, the proportion of undissociated acetic acid molecules is increased, as its ionisation is suppressed. As the acetates of the alkali metals are ionised to a great extent in solution, the concentration of acetate ions can be increased by adding them to the solution ; while the hydrion concentration can be made larger by the addition of strong acids, such as hydrochloric acid. In general, the ionisation of a weak electrolyte is reduced by adding a strong electrolyte with a common ion. Compared with it, the converse effect of the weak electrolyte on the ionisation of the strong electrolyte completely disappears.

Such mixtures of a weak electrolyte with a corresponding strong electrolyte with a common ion, *e.g.*, acetic acid—acetate mixture, or the combination ammonium hydroxide—ammonium chloride, have an important distinctive property which is

* H. Lundén, "Affinitätsmessungen an schwachen Säuren u. Basen." Stuttgart. F. Enke. 1908. (Literature.)

brought out clearly by solving the dissociation equation as follows:—

(1) $$C_{H^{\cdot}} = K \frac{C_{CH_3.COOH}}{C_{CH_3COO'}}.$$

(2) $$C_{OH'} = K' \frac{C_{NH_4OH}}{C_{NH_4^{\cdot}}}.$$

In an acetate—acetic acid mixture, the ionisation of the acetic acid, which is low to begin with, is so far suppressed that the acetate ions in solution can be regarded in practice as derived solely from the acetate. As the acetate is ionised to more than 90 per cent. in solutions below 0·01 N, the concentration of the acetate can be written instead of that of the ion. Further, the concentration of the un-ionised molecules of acetic acid is practically identical with that of the acetic acid actually used. So that—

$$C_H = K \frac{C_{\text{acetic acid}}}{C_{\text{acetate}}} \qquad [K_{18^\circ} = 1{\cdot}8 \times 10^{-5}],$$

and similarly for the base,

$$C_{OH} = K' \frac{C_{\text{ammonia}}}{C_{\text{ammon.chloride}}} \qquad [K' = 1{\cdot}77 \times 10^{-5}].$$

It is easy to see that the mere proportion of acid (or base) and salt determines the concentration of $H^{\cdot}$ or OH' ions; and as this proportion is independent of dilution over a considerable range, mixtures of weak acids and bases with highly dissociated salts provide a simple means of preparing in practice media of known $H^{\cdot}$ or OH' concentration. Such solutions are readily reproducible, and proof against reasonable impurities or alterations in volume. They are called regulators or *buffer solutions*. The table on p. 26 gives an example of the ion concentrations in such solutions.

This relation between weak and strong electrolytes with a common ion is of importance in the determination of the iso-electric reaction of albumin by means of regulators (L. Michaelis). Further, such solutions are useful for finding out how much of a weak acid or base is combined with albumin, and for estimating the hydrolysis of the salt-like protein compounds formed in this way (Wo. Pauli and Hirschfeld, see Chapter V.).

Table 4.

(*Valid for* N/100 *and more dilute solutions.*)

Acetic acid: acetate.	$C_{H^\cdot}$	Acetic acid: acetate.	$C_{H^\cdot}$	$\frac{NH_4Cl}{NH_4OH}$	$C^*_{H^\cdot}$ (18°)	$\frac{NH_4Cl}{NH_4OH}$	$C^*_{H^\cdot}$ (18°)
32/1	$5{\cdot}76 \times 10^{-4}$	1/2	$0{\cdot}9 \times 10^{-5}$	32/1	$1{\cdot}02 \times 10^{-8}$	1/1	$0{\cdot}32 \times 10^{-9}$
16/1	$2{\cdot}88 \times 10^{-4}$	1/4	$0{\cdot}45 \times 10^{-5}$	16/1	$0{\cdot}51 \times 10^{-8}$	1/2	$0{\cdot}16 \times 10^{-9}$
8/1	$1{\cdot}44 \times 10^{-4}$	1/8	$0{\cdot}22 \times 10^{-5}$	8/1	$0{\cdot}26 \times 10^{-8}$	1/4	$0{\cdot}8 \times 10^{-10}$
4/1	$0{\cdot}72 \times 10^{-4}$	1/16	$0{\cdot}11 \times 10^{-5}$	4/1	$0{\cdot}13 \times 10^{-8}$	1/8	$0{\cdot}4 \times 10^{-10}$
2/1	$0{\cdot}36 \times 10^{-4}$	1/32	$0{\cdot}56 \times 10^{-6}$	2/1	$0{\cdot}64 \times 10^{-9}$	1/16	$0{\cdot}2 \times 10^{-10}$
1/1	$1{\cdot}8 \times 10^{-5}$	1/64	$0{\cdot}28 \times 10^{-6}$	—	—	1/32	$1{\cdot}00 \times 10^{-11}$

* From the dissociation equation of water $C_{H^\cdot} \times C_{OH'} = K_{w^\cdot}$ ($K_w = 0{\cdot}58 \times 10^{-14}$ at 18°).

The proteins, as we have emphasised above, are amphoteric electrolytes, behaving both as acids and as bases. They have both an acid dissociation constant K_a and a basic dissociation constant K_b, or, should they be poly-basic acids or poly-acid bases, more than one dissociation constant of each kind. We will not here discuss the observations so far made, but these show that if certain conditions for the determination of the iso-electric reaction are observed, a single mean constant K_a can be taken as an index of the acid strength of the protein, and similarly K_b as a measure of the basic character, without encountering any difficulty.

This effect is in complete accordance with the general behaviour of polyvalent acids or bases which dissociate in several stages; for, as a rule, the first stage is large compared with the later ones, as indicated by the greatest development of H· or OH′ ions.

Most of the natural proteins that have been studied, whether simple or compound (albumin, globulin, glutin, casein, hæmoglobin), show a stronger acid than basic character. As, therefore, the dissociation into hydrion predominates, so does the electro-negative character of the colloidal portion of the protein, as is shown by the marked anodic migration of the pure natural substance. It can be predicted from the fact that these proteins are built up of amino-acids, which themselves are more acid

than basic in character, that such bodies will be amphoteric electrolytes with $K_a > K_b$.

The following table gives the dissociation constants of some amino-acids and di-peptides, and, for comparison, those of a few weak acids and bases which are occasionally used :—

Table 5.

Dissociation Constants at 25°

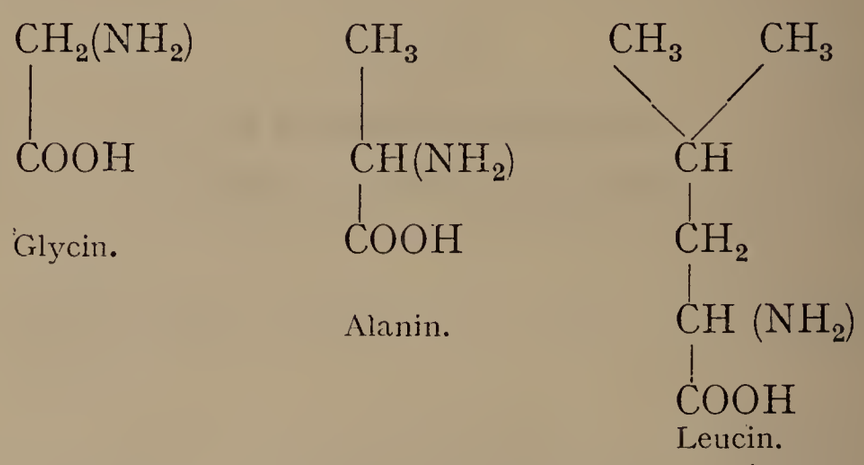
(*when not otherwise stated*).

Substance.	K_a	K_b	$C_{H^{\cdot}} = \sqrt{\frac{K_a \times K_w}{K_b}}$	$K_a \times K_b$
Glycin	$1{\cdot}8 \times 10^{-10}$	$2{\cdot}7 \times 10^{-12}$	$2{\cdot}6 \times 10^{-7}$	$4{\cdot}86 \times 10^{-22}$
α-Alanin	$1{\cdot}9 \times 10^{-10}$	$5{\cdot}1 \times 10^{-12}$	$1{\cdot}9 \times 10^{-7}$	$9{\cdot}96 \times 10^{-22}$
Leucin	$1{\cdot}8 \times 10^{-10}$	$2{\cdot}3 \times 10^{-12}$	$2{\cdot}9 \times 10^{-7}$	$4{\cdot}14 \times 10^{-22}$
Tyrosin	$4{\cdot}0 \times 10^{-9}$	$2{\cdot}6 \times 10^{-12}$	$3{\cdot}9 \times 10^{-6}$	$1{\cdot}04 \times 10^{-20}$
Histidin	$2{\cdot}2 \times 10^{-9}$	$5{\cdot}7 \times 10^{-9}$	$6{\cdot}2 \times 10^{-8}$	$1{\cdot}25 \times 10^{-17}$
Aspartic acid	$1{\cdot}5 \times 10^{-4}$	$1{\cdot}2 \times 10^{-12}$	$1{\cdot}1 \times 10^{-3}$	$1{\cdot}8 \times 10^{-16}$
β-*i* asparagin	$1{\cdot}35 \times 10^{-9}$	$1{\cdot}53 \times 10^{-12}$	—	$2{\cdot}07 \times 10^{-21}$
Methylglycin	$1{\cdot}2 \times 10^{-10}$	$1{\cdot}7 \times 10^{-12}$	—	$2{\cdot}04 \times 10^{-22}$
Dimethylglycin	$1{\cdot}3 \times 10^{-10}$	$9{\cdot}8 \times 10^{-13}$	—	$1{\cdot}27 \times 10^{-22}$
Phenylalanin	$2{\cdot}5 \times 10^{-9}$	$1{\cdot}3 \times 10^{-12}$	$4{\cdot}4 \times 10^{-6}$	$3{\cdot}25 \times 10^{-21}$
Glycylglycin	$1{\cdot}8 \times 10^{-8}$	$2{\cdot}0 \times 10^{-11}$	$3{\cdot}0 \times 10^{-6}$	$3{\cdot}60 \times 10^{-19}$
Alanylglycin	$1{\cdot}8 \times 10^{-8}$	$2{\cdot}1 \times 10^{-11}$	$3{\cdot}0 \times 10^{-6}$	$3{\cdot}60 \times 10^{-19}$
Leucylglycin	$1{\cdot}5 \times 10^{-8}$	$3{\cdot}1 \times 10^{-11}$	$2{\cdot}2 \times 10^{-6}$	$4{\cdot}5 \times 10^{-19}$
Lysin	$2{\cdot}0 \times 10^{-12}$	—	—	—
Succinimide	$2{\cdot}8 \times 10^{-11}$	—	—	—
Betain (trimethylglycin)	—	$7{\cdot}0 \times 10^{-13}$	—	—
Urea	—	$1{\cdot}5 \times 10^{-14}$	—	—
Guanin	—	$8{\cdot}35 \times 10^{-12}$ (40° C.)	—	—
Creatine	—	$1{\cdot}87 \times 10^{-11}$ (40° C.)	—	—
Aniline	—	$4{\cdot}6 \times 10^{-10}$	—	—
Ammonium hydroxide	—	$1{\cdot}87 \times 10^{-5}$	—	—
Acetic acid	$1{\cdot}86 \times 10^{-5}$	—	—	—
Lactic acid	$1{\cdot}38 \times 10^{-4}$	—	—	—
Succinic acid	$6{\cdot}7 \times 10^{-5}$	—	—	—

Without treating the question exhaustively we can discuss on the basis of this table the relation of the dissociation constants to the constitution of the amino-acids, which is not without importance for the proteins.

Glycin, alanin, and leucin have the same K_a, and their slightly different basic dissociation constants are of the same

order of magnitude. We can conclude, after inspecting their constitutional formulæ, that, with the NH_2-group in the α-position, the length or ramifications of the carbon chain have but little influence on the properties of these amphoteric electrolytes.



The introduction of a methyl group in place of the hydrogen of the amino-group causes a slight weakening both of K_a and of K_b in the case of glycocoll. On the other hand, in the series alanin, phenylalanin, *p*-hydroxyphenylalanin (tyrosin), an obvious increase in the acid character can be seen, while the basic properties are but little affected.

The formation of a dipeptide from a simple amino-acid results in a large increase of K_a (by 10^2), and a decrease of K_b (by one power of ten only). The combination of either alanin or leucin with glycin to produce a diglycin produces identical properties in the product. It is improbable that the separation of the amino- and carboxyl-groups, which adjoin in the simple α-amino-acids, is sufficient to account for the alterations in ionisation, but it is quite possible that the acid character of the peptide linkage, especially in the lactim form, may play an essential part. This is in agreement with our experience of its function in the formation of protein salts. The distance between the NH_2 and the COOH groups is the same for glycyl-, leucyl-, and alanyl-glycin, while the rest of the carbon chains, of varied length, become branched. The latter, both in these cases and in the simple amino-acids we have considered, appear to have no influence on the dissociation.

A comparison of the amino-mono- and the amino-di-car-

boxylic acids with the corresponding acids of the normal aliphatic series is of the highest interest. Aspartic acid is related to succinic acid in the same way as glycocoll is related to acetic acid. The relationship becomes still clearer by comparison with the corresponding hydroxy-acids:—

$$\underset{\text{Acetic acid.}}{CH_3COOH} \longrightarrow \underset{\text{Glycollic acid.}}{HO.CH_2.COOH} \longrightarrow \underset{\text{Glycocoll.}}{NH_2.CH_2.COOH}$$

$$\underset{\text{Succinic acid.}}{\begin{array}{l} CH_2.COOH \\ | \\ CH_2.COOH \end{array}} \longrightarrow \underset{\text{Malic acid.}}{\begin{array}{l} HO.CH\,COOH \\ | \\ CH_2COOH \end{array}} \longrightarrow \underset{\text{Aspartic acid.}}{\begin{array}{l} NH_2.CH.COOH \\ | \\ CH_2COOH \end{array}}$$

The introduction of NH_2 into the acetic acid molecule causes a vast decrease in K_a, which drops from $1{\cdot}86 \times 10^{-5}$ to $1{\cdot}8 \times 10^{-10}$. On the other hand, on forming the amino-acid from succinic acid the acid dissociation constant rises from $6{\cdot}7 \times 10^{-5}$ to $1{\cdot}5 \times 10^{-4}$. The basic dissociation due to the amino-group in glycocoll and in aspartic acid does not, however, differ greatly in the two cases. If the carboxyl-group in aspartic acid is transformed into an amido-group asparagin is formed, and K_a falls by 10^5 to about the same order of magnitude as that of the amino-monocarboxylic acids. The basic dissociation is not, however, increased, but remains rather below that of the simple amino-monocarboxylic acids. The basic character of the amino-group introduced is almost completely nullified by the acid amide linkage.

From these examples it can be seen that alterations in K_a and K_b are very varied and relatively independent of known constitutive alterations. Further, it is impossible to conclude that because two amphoteric electrolytes have the same K_a and K_b their constitutions correspond to any great extent.

In order to understand the alterations in state of the proteins, it is of great importance to have an accurate knowledge of their dissociation when in the iso-electric state. The latter state is indicated by a lack of motion in either direction in the electric field, and shows that an equal number of electro-negative and electro-positive particles are present. The following considerations were originally brought forward by L. Michaelis, and further developed from one point of view by

S. P. L. Sörensen, and in applying them to the iso-electric state a restriction must be observed which has not so far been sufficiently emphasised. Two kinds only of ionised protein particles (negative and positive), which have originated exclusively from a dissociation into either $H^{\cdot}$ or OH' ions, must exist in the solution. The importance of this restriction in theory and practical work will appear later on.

For a given concentration of protein (or other amphoteric electrolyte) let K_a and K_b be the acidic and basic dissociation constants, $C_{A^{\cdot}}$ the concentration of anions, $C_{K'}$ of cations, while $C_{H^{\cdot}}$ and $C_{OH'}$ are the concentrations of $H^{\cdot}$ and OH' respectively, and x that of the undissociated portion; then the equilibrium equations are :—

$$C_A \,.\, C_H = K_a \,.\, x \quad \text{(I.)}$$

and

$$C_K \,.\, C_{OH} = K_b \,.\, x \quad \text{(II.)}$$

At the iso-electric point $C_A = C_K$ by definition, and by division I. and II. give for the dissociation equilibrium :—

$$\frac{K_a}{K_b} = \frac{C_H}{C_{OH}} \quad \text{(III.)}$$

The behaviour of the undissociated portion at the iso-electric point is of great practical interest. This portion (x) equals the total concentration (n) less the concentrations of the protein ions, thus :—

$$x = n - C_A - C_K\,;$$

substituting from I. and II.,

$$x = n - \frac{K_a x}{C_H} - \frac{K_b x}{C_{OH}}\,;$$

hence

$$\frac{n}{x} = 1 + \frac{K_a}{C_H} + \frac{K_b}{C_{OH}}$$

and

$$\frac{x}{n} = \rho = \frac{1}{1 + \frac{K_a}{C_H} + \frac{K_b}{C_{OH}}}\,.$$

The undissociated fraction, ρ, is the ratio of the concentration

of neutral particles to the total concentration, and is also the relative measure of the undissociated part of the amphoteric electrolyte. The dissociation equation of water (of which the normality then remains constant at 1000/18) can be applied to aqueous solutions when the concentration is not too high :—

$$C_H \cdot C_{OH} = K_w \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (IV.)$$

Where $K_w = 1{\cdot}1 \times 10^{-14}$ at 25°, and, substituting $\frac{K_w}{C_H}$ for C_{OH}, we get

$$\rho = \frac{1}{1 + \frac{K_a}{C_H} + \frac{K_b \cdot C_H}{K_w}},$$

in which ρ is merely a function of C_H and is at a maximum when the expression

$$1 + \frac{K_a}{C_H} + \frac{K_b \cdot C_H}{K_w} = u$$

has a minimum value. Differentiating, we get

$$\frac{du}{dC_H} = -\frac{K_a}{C_H^2} + \frac{K_b}{K_w},$$

whence the condition for a minimum value, $\frac{du}{dC_H} = 0$, leads to the equation

$$\frac{K_b}{K_w} = \frac{K_a}{C_H^2}.$$

Substituting from IV.,

$$\frac{K_a}{K_b} = \frac{C_H}{C_{OH}} \; *.$$

Thus ρ has a maximum value at the iso-electric point (see equation III.).

The undissociated fraction, that is, the ratio of the number of electrically neutral particles to the total concentration, is at a maximum at the iso-electric point.

If values of ρ and P_H are plotted on a graph, this relation is clearly seen. The low concentrations of hydrions are plotted in negative powers of ten, *i.e.*, $C_H = 10^{-P_H}$. The number P_H ($= -\log_{10} C_H$) is called the hydrion exponent by Sörensen,

* For comparisons with the same protein, the above equations are valid both for molecular and for weight percentage concentrations.

and the use of this notation simplifies both calculations and the construction of the graph. In Fig. 4, the abscissæ are the hydrion exponents, P_H, while the ordinates are the values of ρ, the undissociated fraction. As the highest value of the concentration of neutral particles (x) is when this coincides with the total concentration of the dissolved substance, the maximum value of ρ is 1. For this value all the particles are electrically neutral, and the dissociation is nil.

It is clear that for amphoteric electrolytes with the same value of the product $K_a \cdot K_b$, the $\rho - P_H$ curves will be identical; only the position of the curves will be displaced to the left

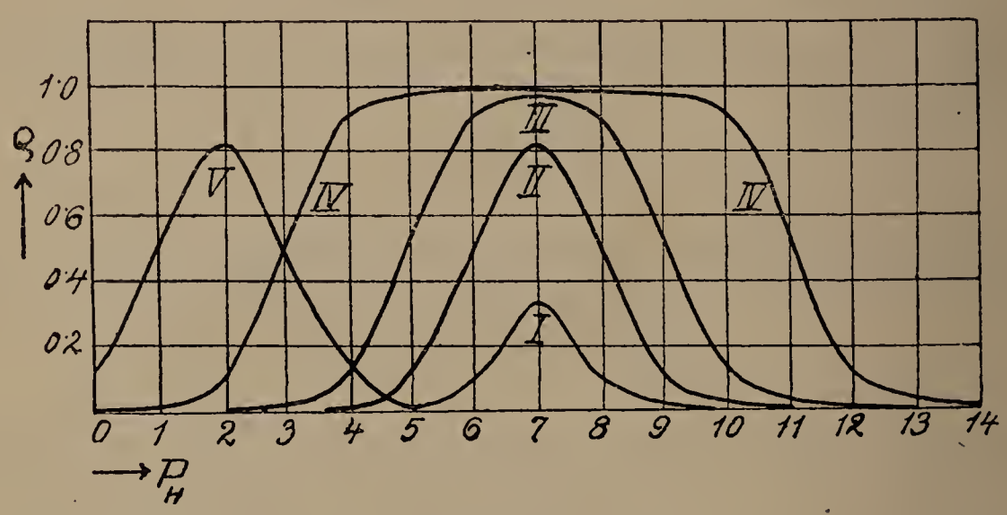


FIG. 4.—Curves of the undissociated fraction (ρ) for $K_w = 1{\cdot}10 \times 10^{-14}$.

with increasing K_a, and to the right with increasing K_b. Curves II. and V. are those for amphoteric electrolytes with product $K_a \cdot K_b = 10^{-16}$. In curve II. $K_a = K_b = 10^{-8}$, while in curve V. $K_a = 10^{-3}$ and $K_b = 10^{-13}$. The maximum proportion of neutral particles, *i.e.*, the iso-electric point, is in the first case (II.) at $P_H = 7$ ($C_H = 10^{-7}$), and in the second case (V.) the increase in K_a shifts it to the left at $P_H = 2$.

The dependence of the form of the curve on the value of the product $K_a \cdot K_b$ is clearly seen in curves I.—IV.

I.	$K_a = K_b = 10^{-7}$	$K_a \cdot K_b = 10^{-14}$
II.	$K_a = K_b = 10^{-8}$	$K_a \cdot K_b = 10^{-16}$
III.	$K_a = K_b = 10^{-9}$	$K_a \cdot K_b = 10^{-18}$
IV.	$K_a = K_b = 10^{-11}$	$K_a \cdot K_b = 10^{-22}$

K_a/K_b is constant for curves I. to IV. and P_H has thus the

same value. The smaller the product $K_a \cdot K_b$ the higher and the more flattened the crest of the curve. When $K_a \cdot K_b = 10^{-18}$, the highest value of the ordinate, $\rho = 1$, is attained, and amphoteric electrolytes with $K_a \cdot K_b < 10^{-18}$ give no sharp iso-electric point, but a more or less wide iso-electric zone, for no definite movement in the electric field occurs over a considerable range of variation of P_H.

When, on the other hand, the value of $K_a \cdot K_b$ increases, the curve rises to a lesser height, and, for a value of $K_a \cdot K_b = 10^{-12}$, reaches a value $\rho = 0$. That is, neutral particles are no longer found, as the amphoteric electrolyte is completely dissociated. Consequently, to obtain a sharply defined iso-electric point, it is necessary to confine the value of the product $K_a \cdot K_b$ between the limits 10^{-18} and 10^{-12}.

The mechanism of the iso-electric reaction on the basis of our deductions and the necessary restrictions can be illustrated as follows. Let x be the neutral particles and A^- and A^+ the anions and cations of an ampholyte due to the H^+ and OH^- ions present. In the case of egg albumin $A^- > A^+$ and the conditions are represented by diagram I. (Fig. 5).

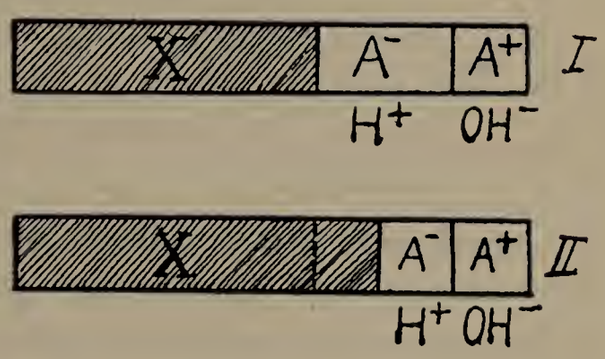


FIG. 5.—Diagram of the iso-electric reaction in buffer solutions.

On addition of acid, the ionisation represented by A^-H^+ is suppressed, until at the iso-electric point $A^- = A^+$, resulting in the effect shown by diagram II. The iso-electric condition is thus characterised by a decrease in the negative ionisation of the albumin A^- and an increase in the number of neutral particles x. The considerations which now follow are valid only for this mechanism of reaction, which, as we shall see later, is not the only possible one.

The determination of the hydrion concentration at the iso-electric point leads directly only to a knowledge of the value of $\frac{K_a}{K_b}$. The aim of a complete electro-chemical characterisation

of natural proteins is, however, the evaluation of the values of both K_a and K_b. The methods employed for measuring these two constants in simpler amphoteric electrolytes, especially those directed to the determination of the dissociation equilibrium of their salts, are scarcely applicable to natural proteins, for reasons which will appear below. More recent attempts to determine K_a and K_b directly by a special method are not yet concluded.

The methods hitherto employed for determining $\frac{K_a}{K_b}$ depend on two principles :—

I. The direct discovery of the iso-electric condition of a protein by means of the application of an electric field in an electrophoresis apparatus, the hydrion content of the solution being varied.

II. The determination of the hydrion concentration which shows, by various tests, a maximum of neutral particles.

We must next make some observations on the first method of determining the iso-electric point by electrophoresis, the discussion of the second method being deferred to the following chapter.*

The technical difficulty of this determination lies mostly in the fact that even with the most precise procedure the change from movement to the anode to movement to the cathode does not occur at a sharply defined point. It more frequently happens that the movement is in both directions (or, less frequently, no movement occurs) over a wide range of hydrion concentration. Sometimes, indeed, in different observations the direction is uncertain. One is therefore forced to take the mean of the limiting values which show definite migration in either sense.

The following facts are important when considering migration in both directions. If the electrophoresis apparatus is filled and left to itself with the taps open, traces of albumin can be

* A third and recent method of determining the hydrion concentration which remains unaltered on varied additions of ampholyte is due to Sörensen, and will be dealt with at the end of the following chapter.

detected above the taps after half an hour, even when no current is applied. This diffusion is more noticeable in concentrated solutions of protein. There is thus a definite tendency for the protein to migrate in both directions, which is affected by an electric field when the protein is charged. The diffusion in one direction is then increased, and in the opposite direction it is decreased. As the iso-electric point is approached, the directive influence of the electric field continually decreases, until conditions finally approximate to those which obtain when no current is passed through the apparatus. The presence of notable quantities of the ions A^- and A^+ in approximately equivalent proportions also has an important effect in the region of the iso-electric point, and may possibly cause migration to a slight extent towards both electrodes.

The products of electrolysis are a frequent source of error when higher concentrations of electrolytes are employed. This disturbing influence can be avoided by the use of unpolarisable electrodes (L. Michaelis), or even in specially favourable cases by preserving a considerable space between the electrodes and the surface of the colloid. A sufficiently large space round the electrodes (Landsteiner and Pauli) and the restriction of the time of passage of the current also decrease the electrolytic effect. Finally, it is necessary, in order to reduce the development of breaks of potential and secondary electrodes in the liquid, to use as connecting liquids solutions of practically the same conductivity and with ions which differ but little in mobility (Pauli and Flecker *). Such secondary electrodes are the chief causes of fluctuations in the sign of the charge in the iso-electric zone, in which, naturally, discharge of the colloid most easily takes place. The following table gives some values for hydrion concentrations (C_H) at the iso-electric point determined by electrophoresis. All the results refer to room temperature (18°). The work on albumin and glutin has been repeated by Pauli and Samec, with identical results. In all cases hydrion regulators, mostly acetic acid—acetate mixtures, were used, but for hæmoglobin, phosphate mixtures and cacodylic acid—sodium salt buffers were also employed.

* *Biochem. Zeitsch.*, 1912, **41**, 995.

Table 6.

Protein.	C_H (found).	C_H (mean).	Author.
Albumin (about 0·6 per cent.).	$1·1—4·2 \times 10^{-5}$	$2·0 \times 10^{-5}$	L. Michaelis and H. Davidsohn, *Biochem. Zeitsch.*, 1911, **33**, 456.
Glutin (0·5 per cent.).	$1·6—3·5 \times 10^{-5}$	$2·5 \times 10^{-5}$	L. Michaelis and W. Grineff, *Biochem. Zeitsch.*, 1912, **41**, 373.
Casein (0·2 per cent.).	$1·29—4·9 \times 10^{-5}$	$2·5 \times 10^{-5}$	L. Michaelis and H. Pechstein, *Biochem. Zeitsch.*, 1912, **47**, 260.
Hæmoglobin .	$0·92—2·8 \times 10^{-7}$	$1·8 \times 10^{-7}$	L. Michaelis and H. Davidsohn, *Biochem. Zeitsch.*, 1912, **41**, 102.

CHAPTER IV

THE PROPERTIES OF PROTEINS IN ISO-ELECTRIC REACTION

THE alterations in properties which natural proteins display in the iso-electric region can, generally speaking, be explained by the difference in physico-chemical behaviour between neutral and ionised protein particles. The relation between the alterations in natural proteins and their electric charge has been worked out in a series of researches by Pauli and his collaborators, with which we shall now deal in detail. We propose briefly to anticipate here such results as appear necessary for the understanding of the discussions which follow.

Neutral albumin shows lower hydration or imbibition, lower viscosity and lower osmotic pressure than ionised albumin. In contrast with electrically charged albumin, it is coagulated both by heat and by the addition of alcohol, and, similarly, only neutral particles form the substratum of coagulation by salts, acids and bases. A small addition of neutral salts stabilises neutral albumin against both spontaneous coagulation and that caused by alcohol or by heating ; whereas it causes ionised albumin to be discharged and dehydrated, and to become sensitive to coagulating agents.

The methods which employ the properties of neutral albumin for the determination of iso-electric behaviour work most simply for those natural proteins of which the neutral particles are not stable in solution owing to their lyophobe character. Casein and the globulins are the most important representatives of this class, and in consequence they show a maximum of precipitation at the iso-electric point. The location of this maximum in mixtures of regulators of varied hydrion concentration is easily carried out. The values given in the following table were obtained in this way.

Table 7.

Maxima of Precipitation.

Substance (under 1 per cent.)	C_H (found).	C_H (mean).	$\frac{K_a}{K_b}$	Author.
Serum globulin . .	$2{\cdot}9—4{\cdot}6 \times 10^{-6}$	$3{\cdot}6 \times 10^{-6}$	$2{\cdot}2 \times 10^{-3}$	L. Michaelis and P. Rona.*
Edestin . .	ca. $1{\cdot}3 \times 10^{-7}$	$1{\cdot}4 \times 10^{-7}$	$2{\cdot}8 \times 10^{0}$	L. Michaelis and P. Rona.
Gliadin . . .	$5{\cdot}8—7{\cdot}5 \times 10^{-10}$	$6{\cdot}0 \times 10^{-10}$	$6{\cdot}0 \times 10^{-5}$	L. Michaelis and P. Rona.
Casein (from milk)	$2{\cdot}0—2{\cdot}8 \times 10^{-5}$	$2{\cdot}4 \times 10^{-5}$		L. Michaelis and H. Pechstein.†

* *Biochem. Zeitsch.*, 1910, **28**, 193.
† *Biochem. Zeitsch.*, 1912, **47**, 260.

The iso-electric point for hydrated natural proteins like albumin and glutin can be found by a greater variety of methods depending on the properties of the neutral particles.

The simplest of these is the method of precipitation by alcohol, as worked out by Pauli in conjunction with J. Matula and M. Samec. By suitable alteration of the protein concentration and of the quantity of alcohol added, an extremely sharp location of the maximum of precipitation is possible.

The following table gives examples from such investigations *

Table 7a.

Ox-serum Albumin.

(1·8 *c.c. alcohol to* 3 *c.c. mixture.*)

Acetic acid : acetate.	C_H	0·2 % albumin.	0·5 % albumin.	1·0 % albumin.	1 % albumin. 30 c.c. albumin + 10 c.c. alcohol. milligrams precipitated
1 : 3	$0{\cdot}6 \times 10^{-5}$	∓ ± Λ	∓ ± Λ	∓ ±	16·1 (no addition).
1 : 2	$0{\cdot}9 \times 10^{-5}$	±	± ±	± ±	17·25
1 : 1	$1{\cdot}8 \times 10^{-5}$	± ± *	± ± ± *	± ± ± *	39·22 *
2 : 1	$3{\cdot}6 \times 10^{-5}$	±	± ±	± ± ±	29·22
3 : 1	$5{\cdot}4 \times 10^{-5}$	— V	— V	∓ ±	19·43
4 : 1	$8{\cdot}2 \times 10^{-5}$	—	+	+	—

* Unpublished results of Pauli and Samec, 1912–13.

on albumin (from ox-serum) and on glutin, both carefully dialysed for many weeks.

Table 8.

Glutin A (*"deutsche Goldmarke"*).

1 *per cent. final concentration* N/100 *acetate.*

C_H (acetic acid—acetate).	Relative co-efficient of viscosity.	Precipitation by alcohol.
$0{\cdot}6 \times 10^{-5}$	1·4954	±
$0{\cdot}9 \times 10^{-5}$	1·4885	± ±
$1{\cdot}8 \times 10^{-5}$	1·4769 *	± ± ± *
$3{\cdot}6 \times 10^{-5}$	1·4977	± ±
$5{\cdot}4 \times 10^{-5}$	1·5509	±
$7{\cdot}2 \times 10^{-5}$	1·5996	—

Acetic acid—acetate mixtures were used as hydrion regulators ; the degree of flocculation is indicated by crosses.*

Experience has shown, further, that the maximum of neutral particles is also expressed in a minimum value of the viscosity, corresponding to the general difference of viscosity between neutral and ionised protein particles.

Fig. 6 (from a research by Pauli and Matula †) shows the depression in viscosity at the iso-electric point in the case of a 1 per cent. glutin at 35°, with acetic acid—acetate mixture.

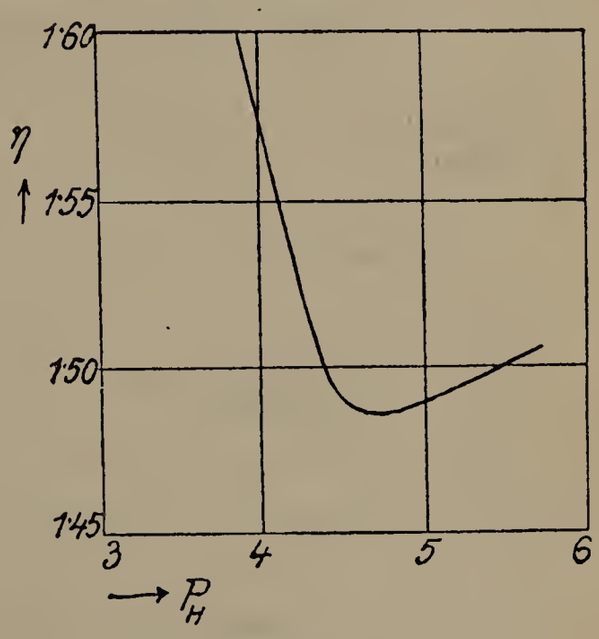


FIG. 6.—Viscosity of glutin at the iso-electric point.

There is good agreement in this instance, as in similar investigations (*e.g.*, with serum containing globulin), between the viscosity minimum and the

* ± slight, ± ± deeper, ± ± ± very deep turbidity. The signs +, + +, + + +, indicate visible formation of precipitate of the same degrees of opacity.

† *Kolloid Zeitsch.*, 1913, **12**, 222.

maximum of precipitation by alcohol. Further, the P_H corresponding to these points agrees satisfactorily with the iso-electric point determined by electrophoresis.

Finally, the osmotic pressure of a protein in the presence of a buffer solution shows a clear and easily reproducible minimum at the iso-electric point. As the ionic dissociation of the protein particles produces an increase of the osmotically effective concentration under the ordinary conditions of experiment, so the formation of neutral particles, at the expense of the ionised particles, causes a diminution in the observed osmotic pressure. As the protein is ionised with liberation of $H^{\cdot}$, metal, or acid ions these kinds of ions are retained by the electrostatic attraction of the colloidal protein ions ; the latter cannot penetrate the osmotic membrane. The imbibition of glutin discs is analogous to the osmotic behaviour.

Table 9.

Glutin 0·5 per cent., N/100 Acetate.

Acetic acid : acetate.	Migration.	Osmotic pressure in millimetre water.	Precipitation by alcohol.
0	To anode . . .	106	—
0 : 1	,, ...	73	—
1 : 4	,, ...	50	—
1 : 3	,, ...	—	±
1 : 2	Changing—amphoteric .	44 *	± ± ± *
1 : 1	To cathode . . .	49	±
2 : 1	,, . . .	—	±
3 : 1	,, . . .	—	—

Imbibition of 5 per cent. gelatin.

Acetic acid : acetate.	Dialysed for three days. Per cent. water taken up.
1 : 3	16·9
1 : 2	9·5 *
1 : 1	10·5
2 : 1	36·8
3 : 1	53·1

The theory of the iso-electric reaction due to L. Michaelis * implies, as already emphasised, the assumption that only two kinds of ions, A^+ and A^-, are present, arising from the dissociation of OH^- or H^+ respectively. The following equations then hold good:—

$$K_a = \frac{[A^-] \cdot C_H}{x} \quad \text{and} \quad K_b = \frac{[A^+] \cdot C_{OH'}}{x}.$$

At the iso-electric point $A^+ = A^-$ and $C_{OH} = \frac{K_w}{C_H}$, so that the hydrion concentration is

$$C_H = \sqrt{\frac{K_a \cdot K_w}{K_b}}.$$

Consequently, the hydrion concentration at the iso-electric point should, at a given temperature, be a constant, quite independent of the dilution of the albumin. The work of Michaelis and of Pauli and Samec on electrophoresis, as also that of the two latter on precipitation by alcohol, does actually show that the hydrion concentration is independent of the content of protein over a considerable range (Table 7a). An experimental proof of this rule exists, however, only when acetic acid—acetate mixture is used, whereas the theory of the iso-electric reaction was extended by its author to the case of any acid, regarding the hydrion concentration present as alone decisive. Sörensen † has formulated this view as follows :—

"To produce in an aqueous solution of an ampholyte the hydrion concentration corresponding to the iso-electric point, so much acid must be added to it (if $K_a > K_b$) that the concentration of acid equals the difference between hydrion and hydroxyl ion concentrations at the iso-electric point. The quantity of acid is *therefore independent of the ampholyte concentration, and is the same as the amount required to impart to pure water the hydrion concentration existing at the iso-electric point of the substance in question.*" L. Michaelis ‡ states quite generally: "If a soluble ampholyte is added to a solution of definite hydrion concentration, it behaves like any acid when the [H·] is less than that at

* See "Handbuch der Biochem." Supplement, p. 24 ff (Jena, 1913), and S. P. L. Sörensen, *Ergebn. der Physiol.*, 1912, **12,** 503.

† *Ergebn. der Physiol.*, 1912, **12,** 503.

‡ *Biochem. Zeitsch.*, 1912, **47,** 251.

the iso-electric point, and as a base if the [H·] is greater than that value. In other words, the ampholyte, like any acid, increases [H·] in the first case quoted ; in the second, like any base, it reduces the [H·]. If, however, the original [H·] is just equal to that at the iso-electric point the added ampholyte produces no change in its value." It remains now to be shown that these considerations do not hold good for strong acids and albumins free from salts (Pauli and Samec *). These authors found, in fact, that on addition of a strong acid a concentration was reached at which a maximum of neutral particles was present, which could be demonstrated both by viscosity measurements and by the alcohol precipitation. This point, however, unlike that obtained by the use of buffer solutions, is largely dependent on the albumin concentration, and in less degree on the nature of the acid. The examples given below and on p. 43 demonstrate this.

The determination of the osmotic pressure also shows the existence of a minimum value. The osmotic pressure is measured against an aqueous solution of the same acid content as the original mixture of acid and albumin put into the osmotic cell. During the twenty-four hours required for the pressure to become constant, acid enters the cell from the outer vessel,

Table 10.

Viscosity of Glutin + Sulphuric Acid at 35°.

$$\frac{t}{t_0} = \frac{\textit{Time of flow of the solution}}{\textit{Time of flow of water}}.$$

Concentration of acid.	Glutin 1·09%. t/t_0.	0·659%. t/t_0.	0·33%. t/t_0.
0	1·458	1·248	1·113
5×10^{-5}	—	1·241	1·111
1×10^{-4}	1·453	1·237	1·109 *
2×10^{-4}	1·445	1·233 *	1·111
$2{\cdot}5 \times 10^{-4}$	1·443 *	1·234	1·113
4×10^{-4}	1·448	1·241	1·118
5×10^{-4}	1·453	1·245	—

* Unpublished researches during 1912–13.

Table 11.

Viscosity of Ox-serum Albumin (final concentration, 1·07 per cent.) with various Acids at 25°.

Concentration of acid.	t/t_0 Hydrochloric acid.	t/t_0 Sulphuric acid.	t/t_0 Oxalic acid.
0	1·063	1·063	1·063
$2·5 \times 10^{-4}$	1·061	1·058	1·061
5×10^{-4}	1·059	1·055	—
$5·6 \times 10^{-4}$	1·057	—	1·057
$8·5 \times 10^{-4}$	1·054 *	1·052	1·054
1×10^{-3}	1·055	1·050 *	1·053
$1·25 \times 10^{-3}$	—	1·050	1·051 *
$1·67 \times 10^{-3}$	1·064	1·053	1·056
$2·1 \times 10^{-3}$	1·068	1·057	—
$2·5 \times 10^{-3}$	1·076	1·064	1·065

Table 12.

Precipitation of Ox-serum Albumin by Alcohol in presence of Sulphuric Acid. (0·5 *c.c. absolute alcohol was added to* 3 *c.c. of the albumin—sulphuric acid mixture.*)

Concentration of acid.	Albumin concentrations.						
	1·1% t/t_0 at 25°.	1·1%.	0·55%.	0·34%.	0·26%.	0·205%.	0·17%.
5×10^{-5} N	—	±	±	∓	±	∓	±
$6·25 \times 10^{-5}$ N	—	±	±	±	±	±	+*
1×10^{-4} N	—	±	±	±	+*	+*	±
2×10^{-4} N	—	+	+	+*	+	±	±
$2·5 \times 10^{-4}$ N	1·047	±±	±±*	±	±	±	—
5×10^{-4} N	1·043	±±	+	±	±	—	—
1×10^{-3} N	1·038 *	±±±*	+	—	—	—	—
$1·25 \times 10^{-3}$ N	1·036	±±	±	—	—	—	—
$1·5 \times 10^{-3}$ N	1·040	±±	—	—	—	—	—
$1·67 \times 10^{-3}$ N	1·042	±	—	—	—	—	—
$2·0 \times 10^{-3}$ N	1·048	±	—	—	—	—	—
$2·5 \times 10^{-3}$ N	1·056	±	—	—	—	—	—

The relative viscosity of a 1·1 *per cent. albumin solution is given for comparison in the second column.*

and the acid content in the osmometer must be ascertained by determining the chlorine present analytically or by an electrometric method. In the following investigation a concentration

of hydrion of $1{\cdot}5 \times 10^{-6}$ was found when the pressure was at a minimum.

Table 13.

Osmotic Pressure of 1 per cent. Albumin at 18°.

Original concentration of HCl.	Osmotic pressure in millimetres of water.	C_H in the osmometer.	C_{Cl} in the osmometer.
0	100	—	—
$7{\cdot}5 \times 10^{-5}$	92	—	—
10×10^{-5}	83	—	—
$12{\cdot}5 \times 10^{-5}$	42 *	$1{\cdot}47 \times 10^{-6}$	$5{\cdot}73 \times 10^{-4}$
15×10^{-5}	58	—	—

A more complete series for glutin gave the following results :—

Table 14.

Osmotic Pressure of 1 per cent. Glutin at 25°.

Original concentration of HCl.	Osmotic pressure in millimetres of water.	Final Concentrations in osmometer. [H·].	[Cl′].
0	126	—	—
$7{\cdot}5 \times 10^{-5}$	87·5	$1{\cdot}32 \times 10^{-6}$	$2{\cdot}82 \times 10^{-4}$
10×10^{-5}	76	$2{\cdot}0 \times 10^{-6}$	$2{\cdot}89 \times 10^{-4}$
$12{\cdot}5 \times 10^{-5}$	70 *	* $3{\cdot}0 \times 10^{-6}$	* $3{\cdot}83 \times 10^{-4}$
15×10^{-5}	82	$5{\cdot}3 \times 10^{-6}$	$4{\cdot}3 \times 10^{-4}$

At the point of maximum precipitation of albumin by alcohol (or of maximum fluidity) a hydrion concentration of $1{\cdot}95 \times 10^{-6}$ with a concentration of chlorine $> 10^{-4}$ was found to obtain.*

* In a later research of Pauli and T. Oryng (*Biochem. Zeitsch.*, 1915, **76,** 373) it is shown that using 1 per cent. serum albumin in a calomel electrode for determining the concentration of chlorions, a chlorine concentration of about 3×10^{-4} normal is developed compared with the solubility of mercurous chloride in water, 1×10^{-5} N. This increase in [Cl′] is due to formation of complex chlorides by the calomel and the albumin. On addition of acid, the mercurous ion is displaced from the albumin, and hydrion takes its place. In the above case we should equate the chlorion concentration, which arises from the salt formation between the HCl and the protein, to the normality of the acid added. For the hydrion is practically completely combined, while the

It is certain from this work by Pauli and Samec that when strong acids are added to albumin or glutin these proteins show a number of properties which agree with those found by means of buffer solutions at the iso-electric point. The hydrion concentration at which this effect occurs is, however, far below that determined by acetic acid—acetate mixtures for the iso-electric point. This is accompanied by a relatively large quantity of acid entering into combination—for 1 per cent. albumin or glutin it is of the order of 10^{-4} N/1.

The equation of electrical neutrality in which, as is well known, the sum of positive ionic charges is equal to the sum of negative ionic charges in any given mixture of electrolytes, for the point of charge in precipitation by alcohol and in viscosity found in the above investigations is as follows :—

$$C_{Cl^-} + C_{OH^-} + C_{A^-} = C_{K^+} + C_{H^+}$$

or, inserting the values in powers of ten which have been determined :—

$$10^{-4}\,(Cl') + 10^{-8}\,(OH') + C_{A^-} = C_{A^+} + 10^{-6}\,(H\cdot).$$

In any case, at this point the concentration of negatively charged albumin must be much reduced compared with the positively charged portion. In fact, in electrophoresis experiments in this region, migration to the cathode is exclusively observed (Table 15). *With strong acids, therefore, the maximum of neutral particles does not coincide with the point of iso-electric migration.*

Apparently, with strong acids, in lower concentration than the P_H of the iso-electric point found by buffer solutions, a regular combination with the albumin occurs. This combination increases with further addition of acid with formation of new positive albumin ions.

One can conclude theoretically that in this case also a particular acid concentration must exist at which the originally anodic migration changes to a cathodic migration, giving rise

chlorion remains completely dissociated not only at such concentrations, but even with much higher content of HCl in a 5 per cent. albumin solution. In the proof appended of the extinction of the positive albumin ions, the chief emphasis must be laid on the observed migration to the positive pole rather than on the excess of chlorine ions present.

to iso-electric effects. This change point can also be demonstrated in the electrophoresis apparatus. For glutin the concentration of added acid which produces this effect lies between 7×10^{-5} and 10×10^{-5} N/1 HCl. The following table gives some collected results for glutin B.

Table 15.

Electrophoresis of Glutin (1 per cent.).

(*75 volts for one hour at* 35°.)

N . HCl.	Migration.
0	To anode.
$2{\cdot}5 \times 10^{-5}$	,,
5×10^{-5}	,,
$7{\cdot}5 \times 10^{-5}$	,,
10×10^{-5}	To cathode.

For the same sample of glutin the maximum number of neutral particles occurs in 1×10^{-3} N.HCl.

Table 16.

Precipitation and Viscosity of 0·5 per cent. Glutin.

N . HCl.	Relative Viscosity at 35°.	Saturation with toluene.	10 c.c. solution + 3 c.c. alcohol.	Depth of the centrifuged precipitate in millimetres.
0	1·285	—	—	0
$0{\cdot}5 \times 10^{-3}$	1·245	± ∧	± ± ∧	negligibly small.
1×10^{-3}	1·213	+ + *	± ± ± *	35 *
$1{\cdot}25 \times 10^{-3}$	1·204 *	± ∨	+ ± ± ∨	24
$1{\cdot}5 \times 10^{-3}$	1·215	±	+ +	21
2×10^{-3}	1·231	—	± ±	19

At the point of iso-electric migration the chlorion concentration is certainly from $7—10 \times 10^{-5}$ N. This is only a minimum value, in view of the possibility of slight diffusion into the albumin from the acid of the same dilution which lies above it in the electrophoresis apparatus. In this protein-acid mixture the hydrion concentration is at most 10^{-6} N. In any case

there occurs at the change point in electrophoresis a considerable combination of acid and consequent formation of electropositive albumin ions. So that a much larger addition of acid is necessary than the iso-electric reaction found by Sörensen and Michaelis would lead us to expect. The considerations developed by these authors for ampholytes in general, therefore, do not hold for proteins and strong acids. The relatively simple results obtained by the use of hydrion regulators containing weak acids exhibit only a particularly favourable limiting case, which varies progressively.

Any theoretical expression of the *reactions of albumin with strong acids* in low concentration must accordingly take into account the following facts :—

1. Addition of strong acids to albumin gives a point of symmetrical electrophoresis and apparently iso-electric behaviour.
2. In these mixtures the concentration of hydrion is very low, and much smaller than that which obtains at the iso-electric point as found by acetic acid—acetate buffer solutions. Thus for 1 per cent. glutin and albumin it is of the order of 10^{-6}.
3. The negative ion of the acid is abundantly present and free in this range of concentration, and there is an excess of electro-positively charged albumin over that which is negatively charged.
4. Further addition of acid produces a maximum of neutral particles, as shown by maximum precipitation by alcohol and minimum viscosity, the electrophoresis being entirely to the cathode in this region.
5. The quantity of acid necessary to produce symmetrical electrophoresis or maximum of neutral particles increases with a rise in the albumin content.

A comprehensive view of the complicated relations governing such cases may be gathered from the following considerations. The strong combination of acid with the protein is certainly demonstrated and must be due to development of activity of basic valencies of the protein which do not function in the presence of very weak acids such as exist in acetic acid—acetate

mixtures. In other words, the mean basic dissociation constant K_b is increased. The hydrion concentration necessary for the development of iso-electric behaviour is decreased by this increase in K_b. Such an effect is actually observed in the results given above for the effect of adding strong acids.

The so-called iso-electric behaviour culminates in the migration of equal quantities of albumin in both directions, or by symmetrical repulsion, when an electric field is applied. This result, however, only proves an equal volume concentration of positively and negatively charged quantities of protein provided that the electro-positive and electro-negative protein particles transported *have the same valency*. For, suppose the positive particles are n-valent, and the negative ones monovalent, then the same weight contains n positive charges, but only one negative charge, so that when one unit of electricity is transferred, the quantity of albumin which migrates towards the negative pole is $\frac{1}{n}$th of the quantity which goes towards the positive pole. The quantity of albumin transported in each direction is the same only when n times as many positive charges are brought by the albumin to the cathode as there are negative charges brought to the anode.

We can, by this time, follow the main lines of the complex mechanism of the reaction of albumin with strong acids if three principles which are in agreement with all previous investigations are grasped.

1. When a strong acid is added it reacts with the basic part of the albumin A^+OH in accordance with the equation, for example :

$$A^+OH + HCl \longrightarrow A^+Cl + H_2O.$$

If the acid is strong enough, it reacts with further basic valencies of the protein when more acid is added, thus :

$$A^+Cl + HCl \longrightarrow A^{++}Cl_2,$$

forming polyvalent ions. (See the next Chapter.)

2. Increase in $[H^\cdot]$ represses the ionisation of the acid portion of the protein A^-H with formation of neutral particles, which can, however, react by means of their basic valencies when more acid is added.

3. The ions formed from pure albumin and weak acids and bases can be considered monovalent in contra-distinction to those of the protein salts.

The addition of strong acids to albumin gives rise to four different effects in the solution which are shown by the points *a, b, c, d* (Fig. 7). In illustration of these we shall adopt the scheme previously employed, and, to simplify notation, choose the reaction with hydrochloric acid as an example.

a. The first point which is reached when acid is gradually added is due to the reaction of $A^{+}OH$ with HCl and a slight repression of the ionisation of $A^{-}H$, so that hydrions and chlorions are present in equal numbers, as also are the charges on the negative and positive albumin.* This is a true iso-electric point which, however, can only be found by an electrometric determination of [H·] and [Cl′]. It occurs at such a low concentration of acid that the small concentration of chlorions cannot be measured. Under these conditions the protein migrates to the anode.

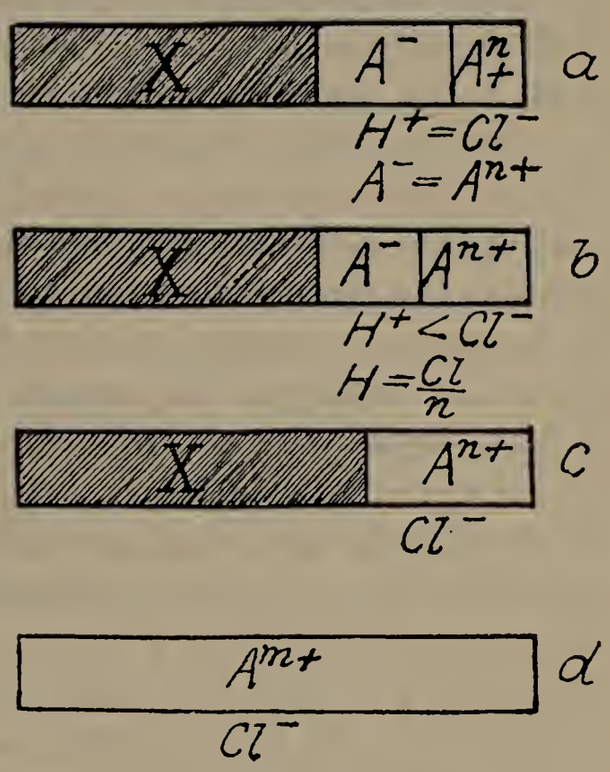


FIG. 7.
a, true iso-electric point.
b, point of iso-molar migration.
c, the maximum of neutral particles.
d, maximum combination with acid.

b. As more acid is added a further point is reached, when the albumin changes its direction of migration and moves towards the cathode instead of towards the anode. This change point in electrophoresis is only apparently iso-electric, as proved by electrometric measurements, which show an excess of negative Cl′ ions over the positive H· ions, the latter having a concentration of 2×10^{-6} the former of $0{\cdot}7$—$1{\cdot}0 \times 10^{-4}$. One is

* The OH′ ions which are present according to the equation $C_H \cdot C_{OH} = K_w$ are here omitted.

forced to the conclusion that the same mass of albumin carries many more positive than negative charges, so that the valency of the positive albumin ion must be assumed to be relatively high. This point can therefore be described as *iso-molar* with reference to electric transport, and as *aniso-electric* with respect to the albumin ions.

c. Still further addition of acid produces purely cathodic migration of the albumin, but at the same time the number of neutral particles reaches a maximum, as is shown by a maximum in the quantity precipitated by alcohol, and a minimum of viscosity, which occurs at approximately the same point. The [Cl′] now exceeds $1{\cdot}0 \times 10^{-3}$, but the [H·] is still 2×10^{-6}. Taking into consideration the equation of electrical neutrality and also the strong migration to the cathode, we conclude that the negative ionisation of the albumin is repressed almost to extinction owing to the formation of neutral particles, which indicate their existence by their precipitation by alcohol and by the decrease in viscosity. The minimum osmotic pressure, however, does not occur at the point *c* of maximum neutral particles, but at an acid concentration which lies between *b* and *c*, at which [Cl′] for 1 per cent. glutin is $3{\cdot}8 \times 10^{-4}$ and for albumin $5{\cdot}7 \times 10^{-4}$ (see Tables 13 and 14). The explanation of this lies in the fact that the ionisation of $A^{n+}.nCl$ gives rise to an increase in osmotic pressure which counteracts, and finally exceeds, the decrease caused by the formation of neutral particles. In consequence of this effect, the pressure minimum lies at an acid concentration below that represented by the point *c*.

d. Above the point *c*, further quantities of acid convert the remaining neutral albumin into acid albumin up to the point of saturation of the albumin with acid, when a maximum of ionisation represented by *d* is reached. The properties of the acid albumin in this zone will be discussed in the next chapter.

The rough diagrams of Fig. 7 serve to illustrate the points at which the properties of acid albumin undergo change. It will be easily understood from them that, with this mechanism of reaction, the quantity of acid required to reach one of these points depends on the albumin concentration; it will be

equally clear that, owing to the almost complete combination with the acid which occurs, the free H· ions, over a wide range preserve a low concentration, which fluctuates only between narrow limits.

On account of the differing valency of the negative groups present, and of the newly-formed positive albumin ions, points which are apparently iso-electric appear when considerable combination with acid occurs. These points have many properties in common with the true iso-electric points found by using hydrion regulators, but they must be clearly distinguished therefrom. The main difference between the two cases lies in the fact that the first three points (*a*, *b*, *c*) are merged into a single point when regulators are used, whereas with strong acids they lie wide apart. Nevertheless, the difference must not be considered absolute. With regulator mixtures, which greatly reduce the ionisation of the acid employed, the formation of polyvalent albumin ions is largely prevented. Consequently there is to some extent a satisfactory agreement between the properties of albumin and those required by the theory of Michaelis, which holds strictly only for amphoytes of simple structure.*

S. P. L. Sörensen† has quite recently made use of the rule that the quantities of acid or base required to make a solution iso-electric are independent of the concentration of ampholyte as a means of determining the iso-electric H· ion concentration. To this end a concentration of H· ion was sought which remained unaltered on addition of any arbitrary quantity of amphoteric electrolyte. A close examination of Sörensen's conditions of work is necessary in order to test how far his results can be reconciled with the quite contradictory ones obtained in the case of strong acids. Sörensen used for his experiments egg

* From this point of view, the importance of the fact that slight variations in the behaviour of the components of the buffer mixture produce small divergences from the point of change of the properties of the protein is brought out. These variations show a certain conformity to those larger differences shown when strong acids are used. Moreover, the hydrion concentration which causes minimum viscosity is regularly slightly greater than that at which the precipitation by alcohol shows a maximum.

† *Zeitsch. physiol. Chem.*, 1918, **103,** 192. See also A. Hasselbalch, *Biochem. Zeitsch.*, 1916, **78,** 129.

albumin, purified with special care by repeated recrystallisation and dialysis.

Consider a series of mixtures with the same content of ammonium sulphate, but containing increasing quantities of sulphuric acid. We get from these a curve in which the free H· ion content (*h*, the abscissæ) increases with the excess of sulphuric acid present (*t*, the ordinates). Suppose we now choose such a mixture that the curve ($e = 0$, in Fig. 8) contains the iso-electric concentration of H· ions for egg albumin. At

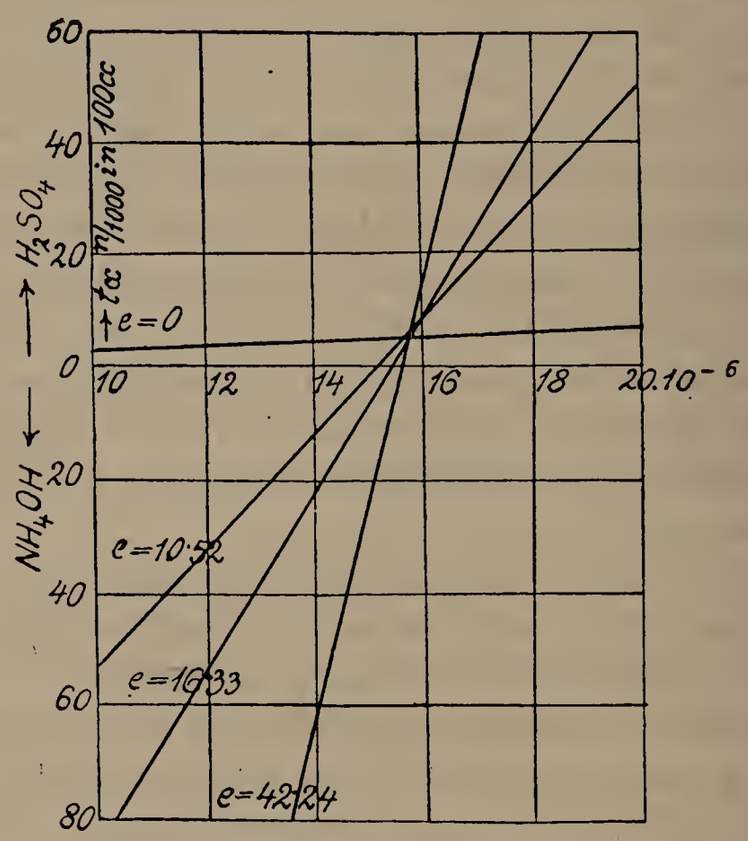


FIG. 8.—Ammonium sulphate concentration = 0·36 N in all cases.
e = milligram equivalents of protein nitrogen in 100 c.c.

this point, the hydrion concentration produced by the same concentration of ammonium sulphate and the same excess of acid should remain unaltered by the presence of egg albumin of any concentration (expressed as *e* milligram equivalents of protein nitrogen). We find instead that the curves obtained for various concentrations of protein intersect the pure ammonium sulphate—sulphuric acid curve, within the limits of

experimental error, at a point representing the same excess of acid and the same hydrion concentration.

Sörensen has made a large number of such determinations with egg albumin, and deduces as a mean value from twenty sets of results, $15{\cdot}74 \times 10^{-6}$ as the iso-electric point of egg albumin. These determinations are undeniably the most accurate investigations of the iso-electric point so far carried out.

From our point of view the following points must be noted :—

This work of Sörensen is another investigation carried out with the use of a weak acid. For, by the presence of ammonium sulphate in the relatively high concentration used in the research quoted above, the strength of sulphuric acid is reduced to one-eighth of its ordinary value. Sörensen himself regards such solutions as mixtures of the acid $H[NH_4SO_4]$ with the salt $NH_4[NH_4SO_4]$.

This work is, therefore, a valuable proof of the fact that if a sufficiently weak acid is used, the iso-electric point is largely independent of the ampholyte concentration. This had already been demonstrated for acetic acid—acetate mixtures by Michaelis, using the method of electrophoresis, and by Pauli by the use of alcohol precipitation and viscometry. The behaviour of proteins when strong acids are employed in the absence of their salts comprises, however, a complex of phenomena quite by itself, as has been previously emphasised.

In pure albumin, as in weak acids and bases in general, a notable dissociation by steps is not to be expected. Consequently it is enough to assume that a single K_a and K_b functions in the buffer solutions. The action of one of the strongest of the acidic and basic groups is sufficient in the first instance to decide the acidic and basic behaviour of the substance. It is easy to understand, in these circumstances, that all differences in constitution do not find expression in the values of K_a and K_b, as has already been seen in the case of the simplest amino-acids and dipeptides. Consequently the iso-electric reaction of various proteins with buffer solutions is only partly affected by differences in structure. Consistent with this view is the scarcely surprising fact that proteins of such widely differing

structure as glutin, albumin and casein show such a very close agreement in the value of the iso-electric concentration (2×10^{-5}) in acetic acid—acetate mixtures. The supposition that, on further combination with acid, a newly-formed positive polyvalent protein ion appears is necessitated by the behaviour of the salts of albumin with acids described in the following chapter.

CHAPTER V

SALTS OF ALBUMIN AND ACIDS

THE reaction of albumin with acid, in a 1 per cent. solution of the former, undergoes a change when the acid concentration exceeds 10^{-3} N. Below that concentration, the evidence for combination is complex, but with greater quantities of acid the process becomes more simple, and reveals itself as the mere saturation of the basic valencies of the protein.

The plain fact that proteins combine both with acids and bases has been known for a long time. On the other hand, quantitative measurements of the extent of combination awaited the clear conception of the terms acid and base, which only physical chemistry could furnish. The results of the application of titrimetric methods to the determination of the capacity of proteins for combination with acids and bases (Spiro and Pemsel) were only made clear by the progressive understanding of the function of indicators (W. Ostwald, H. Friedenthal, E. Salm and others). Certain proteins, such as casein, have been shown to form approximately neutral salts when titrated with strong alkalis, using phenol phthalein as indicator, the point of colour change in this instance being near the true point of neutrality in the physico-chemical sense (Laqueur and Sackur). Many other indicators, such as phloroglucin—vanillin, give an idea of the maximum combination of acids with albumin, which, although only rough, is sufficient for many purposes (J. Christiansen). The work of S. P. L. Sörensen,* in particular, has shown that, in order to arrive at useful conclusions as to hydrion concentrations by the use of indicators, special precautions must be taken when proteins

* *Compt. rend. lab. Carlsberg*, 1907, **7**, 1; 1909, **8**, 1; *Biochem. Zeitsch.*, 1907, **7**, 45; 1909, **21**, 131; 1909, **22**, 352. For summary and literature, see *Ergebn. d. Physiol.*, 1912, **12**, 303; and L. Michaelis, "Die Wasserstoffionenkonzentration." 1914. Berlin. J. Springer.

are present. Our exposition of the combination of albumin with acids, and of the physico-chemical properties of the products formed thereby, will be based mostly on recent researches, which, without disturbing the equilibrium between protein and acid, have led, on the basis of direct determination of hydrion concentrations, to a pretty comprehensive view of the phenomena which occur.

Measurements of electrical conductivity, as undertaken by J. Sjöqvist* in the first instance, give a limited insight into the behaviour of proteins with acids. As long as combination occurs, the conductivity shows a considerable decrease owing to the disappearance of the rapidly-moving hydrogen ions. This, however, is an indirect and uncertain way of arriving at the capacity of the albumin for combination with acid, because, as we shall see, the mobility of the charged albumin particles is in no wise independent of the quantity of acid combined therewith, and, moreover, on account of the high mobility of the hydrogen ion, a slight excess of uncombined acid makes a considerable difference to the conductivity of the mixture.

The important work of F. A. Hofmann and O. Cohnheim,† in which the acceleration of the inversion of cane sugar by hydrogen ions was applied to estimate the hydrion content of mixtures of proteins and acids, only fails on account of the slight sensitiveness of the method in such cases.

The work of St. Bugarsky and L. Liebermann ‡ marks a stage of notable progress in the investigation of the matter under discussion. These authors introduced the measurement of H and Cl ions by the electrometric method to determine the combination of acids, bases, and common salt with proteins. Until recently, this work was one of the main supports of the view of the salt-like nature of the acid and alkali albumins which was commonly accepted. The products were therefore considered to be subject in typical fashion to the laws of electrolytic dissociation and of hydrolysis, which were consequently applied to them.

* *Skand. Arch. Physiol.*, 1894, **5**, 277 ; 1895, **6**, 255.

† O. Cohnheim, *Zeitsch. Biol.*, 1896, **33**, 489 ; see also W. B. Hardy, *J. Physiol.*, 1905, **33**, 251.

‡ *Arch. ges. Physiol.*, 1898, **72**, 51.

Bugarsky and Liebermann worked on egg albumin dialysed free from salts, and on mixtures of albumoses purified in a similar way. Their measurements of the hydrion concentration are, however, more troublesome and less reliable than those more recently carried out by S. P. L. Sörensen and Hasselbalch with much improved technique. The following table gives an example of the results of Bugarsky and Liebermann :—

Table 17.

(*g = gm. albumin to* 100 *cc.* 0·05 *N Hydrochloric Acid.*)

g.	% H combined.	% Cl combined.
0·4	9·0	10·7
0·8	18·9	20·2
1·6	33·3	38·0
3·2	60·2	64·0
6·4	96·56	76·0
12·8	99·67	—

These figures, which are displayed graphically in Fig. 9, show that on addition of more than 6 gm. albumin to 100 c.c. of 0·05 N HCl, the acid combines completely with it. Also, with 3 gm. albumin and lesser quantities, the H· ions and Cl′ ions are taken up in equal number, but above that concentration of protein, the H· ion is more completely combined than the Cl′ ion. The authors deduce from this the constitution of the compound of albumin and acid : " The compound albumin—HCl dissociates in water to give Cl′ ions and albumin—H· ions (the latter might be named ' albuminium ') ;

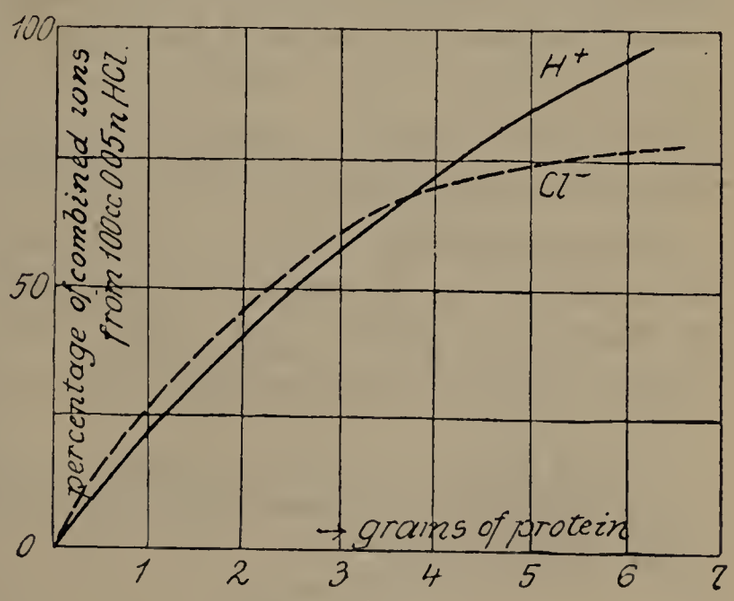


Fig. 9 (Table 17).

hence its constitution is analogous to that of ammonium chloride."

Bugarsky and Liebermann attribute to the albuminium chloride a smaller dissociation than that of ammonium chloride, and hence, as soon as a sufficient excess of free hydrochloric acid is present, as is the case with a lower albumin content, the compound is but little ionised. When, however, much albumin is in solution, and the acid almost completely combined with it, the dissociation into Cl′ ions, which in lower concentration is suppressed, comes into play, giving thus many more Cl′ than H· ions. This thoroughly plausible conclusion has been accepted by most authorities, although it is based solely on the single experiment quoted above. Indeed, it rests entirely on the difference in combination of H· ions and Cl′ ions observed once only at an albumin concentration of 6·4 gm. This view was controverted by T. B. Robertson,* who pointed out the quite inadequate experimental evidence for it, and pronounced the single experiment relied on by Bugarsky and Liebermann as doubtful. Robertson, on his part, supposes that when albumin combines with acids, complete combination with both ions always ensues; and, as the conductivities of such acid—protein mixtures assume such high values, that not merely the small balance of free acid but also the acid albumin must contribute to it. Robertson therefore assumes that fresh positive and negative protein ions are formed, of which the former should contain the H· ion and the latter the Cl′ ion.†

This hypothesis, which is put forward as quite general, we have proved to be certainly inapplicable to the combination of albumin or glutin with acids. It is, in the first instance, contrary to the fundamental fact that cataphoresis experiments

* "The Physical Chemistry of the Proteins." Longman. 1918.

† According to Robertson, albumin reacts with HCl by the operation of the peptide linkage:

$$—CO—NH— \longrightarrow —C(OH) = N—$$

Thus

$$\ldots —C(OH) = N— \ldots + HCl \longrightarrow \ldots —COH^{++} \ldots + \overset{\overset{H}{|}}{\underset{\underset{Cl}{|}}{N''}}— \ldots$$

undertaken with every precaution show that movement to the anode alone occurs in acid albumin, indicating the presence of only positive albumin ions.

The ionisation of albumin-acid compounds has been made quite clear by the careful measurements of H· and Cl′ ion concentrations by the potentiometer method undertaken by K. Manabe and J. Matula.* Their results for an albumin made from ox-blood serum are given below. The arrangement is much clearer than that of Bugarsky and Liebermann, for in this case the albumin concentration (about 1 per cent.) was kept constant, the acid alone being varied.

Table 18 (Fig. 10).

Ox-serum Albumin (end concentration 1·09 per cent.) + HCl.

A. Hydrion Concentrations.

Final concentration of HCl (× N).	P_H.	C_H.	H combined.
0·005	3·410	$3·89 \times 10^{-4}$	$4·48 \times 10^{-3}$
0·007	3·313	$4·75 \times 10^{-4}$	$6·32 \times 10^{-3}$
0·01	3·085	$8·22 \times 10^{-4}$	$8·84 \times 10^{-3}$
0·02	2·295	$5·07 \times 10^{-3}$	$1·41 \times 10^{-2}$
0·03	—	—	$1·52 \times 10^{-2}$ (extrap.)
0·05	1·536	$2·91 \times 10^{-3}$	$1·79 \times 10^{-2}$

B. Chlorion Concentrations.

HCl.	P_{Cl}.	C_{Cl}.	Cl combined.
0·005	2·366	$4·31 \times 10^{-3}$	$5·61 \times 10^{-4}$
0·007	2·340	$4·57 \times 10^{-3}$	$2·23 \times 10^{-3}$
0·01	2·085	$8·22 \times 10^{-3}$	$1·39 \times 10^{-3}$
0·02	1·863	$1·37 \times 10^{-2}$	$5·44 \times 10^{-3}$
0·03	1·678	$2·10 \times 10^{-2}$	$7·54 \times 10^{-3}$
0·05	1·517	$3·04 \times 10^{-2}$	$1·66 \times 10^{-2}$

It can be seen from these results that in lower concentrations of acid practically all the H· ions are combined. With an increase in the quantity of acid the combination tends to a

* *Biochem. Zeitsch.*, 1913, **52**, 369.

maximum, which amounts in this case to $1 \cdot 8 \times 10^{-2}$ N per litre in 1·09 per cent. albumin solution. The Cl′ ions, on the other hand, remain to a large extent free in the beginning, and only above a concentration of 0·02 N acid does the combination of the chlorine increase rapidly. If, now, the difference of the combined H· and Cl′ ions is plotted as ordinates (Fig. 10), a direct view of the behaviour of the ionisation in acid albumin is obtained (the fine dotted curve). The ionisation increases to a maximum in about 0·02 N HCl, and then falls off, until at a concentration of 0·05 N it is almost completely suppressed. This phenomenon indicates that the free acid which accumulates on further acidification, after considerable saturation of the protein finally suppresses almost completely the ionisation of the albumin chloride. It is only this work and similar experiments with serum albumin which proved that the theory of the ionisation of albumin chloride formulated by Bugarsky and Liebermann was based on facts, whereas the alternative conception of T. B. Robertson is definitely refuted.

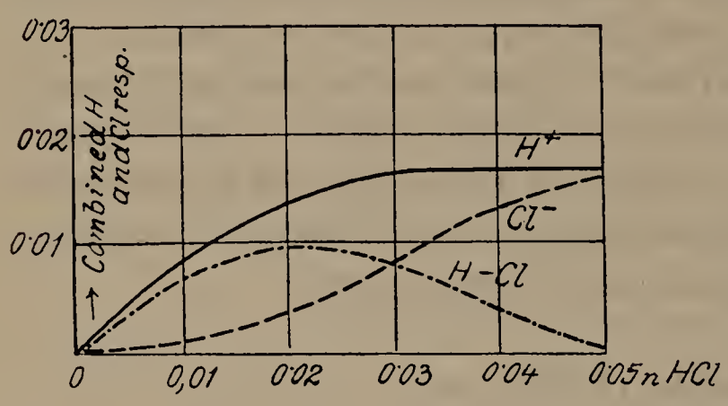


FIG. 10 (Table 18).

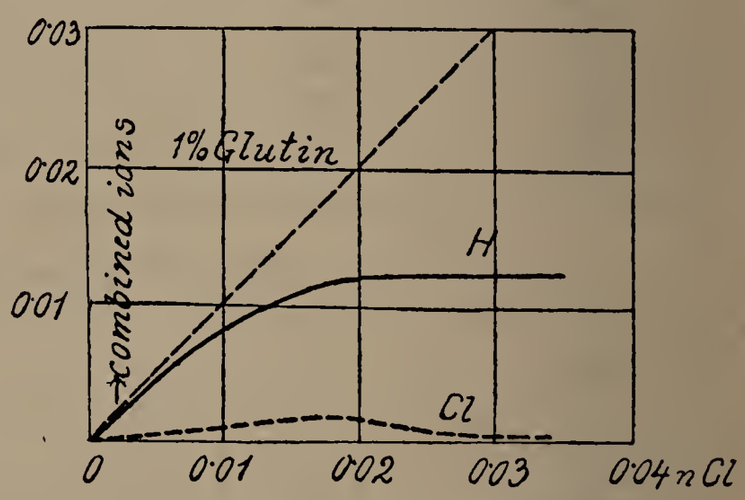


FIG. 11.—Glutin and hydrochloric acid.

Experiments with glutin confirm the main result obtained in the work on albumin, that is, that ionisation of the acid-protein occurs with splitting off of chlorine ions, indeed, in this case the fraction dissociated is still larger than with serum

albumin. The strong tendency to ionisation of the glutin chlorides is indicated by the fact that even with an acid concentration of 0·05 N the dissociation of the protein salt is not notably suppressed. Glutin chloride thus approaches somewhat closely to ammonium chloride in its properties.

Table 19 (Fig. 11).

Glutin (1 per cent.) + HCl.

Final concentration of HCl.	$P_{H^\cdot}$	H combined.	$P_{Cl^\cdot}$	Cl combined.
0·003 N	4·74	$2·9 \times 10^{-3}$	2·50	—
0·005 N	3·97	$4·8 \times 10^{-3}$	2·32	$4·0 \times 10^{-5}$
0·007 N	3·80	$6·6 \times 10^{-3}$	2·19	$3·4 \times 10^{-4}$
0·01 N	3·01	$8·7 \times 10^{-3}$	2·00	—
0·015 N	2·44	$10·8 \times 10^{-3}$	1·89	$1·8 \times 10^{-3}$
0·02 N	2·10	$11·1 \times 10^{-2}$	1·74	$1·1 \times 10^{-3}$
0·03 N	—	—	1·53	—
0·05 N	—	—	1·31	—

The results of varying the protein content with a constant concentration of acid can now be better understood. It is clear that, at a low concentration of albumin, with a given content of acid, relatively more H· ions will be combined than when more albumin is present, owing to the fact that up to a point excess of acid favours the combination of the H· ions (curve H, Fig. 12). Further, as the combination of the Cl′ ions always lags behind that of the H· ions, the absolute quantity of combined Cl′ ions gradually rises to a maximum with increasing concentrations of protein, and then rapidly decreases (see curve II., Fig. 10).

Table 20 (Fig. 12).

Serum Albumin (various concentrations) + 0·02 N HCl.

Concentration of albumin (%).	H combined.	Cl combined.
0·46	$7·26 \times 10^{-3}$	$3·31 \times 10^{-3}$
0·92	$11·3 \times 10^{-3}$	$5·30 \times 10^{-3}$
1·37	$13·5 \times 10^{-3}$	$7·32 \times 10^{-3}$
1·73	$16·4 \times 10^{-3}$	$5·95 \times 10^{-3}$

The natural explanation of these relations is that with low content of albumin at constant acidity the behaviour of the H· and Cl′ ions approaches that which obtains in presence of excess of acid, whereas when much albumin is present the behaviour is akin to that of complete combination of the acid. When the same series of determinations is made at higher concentrations of acid, the divergence of the curves is displaced in the direction of the higher concentration of albumin (Fig. 12). At the same time these investigations lead to the observation that the measurements of Bugarsky and Liebermann give too high a value for the free H· ions, and consequently the figures for combined acid are too low (compare Fig. 12 and Fig. 9).

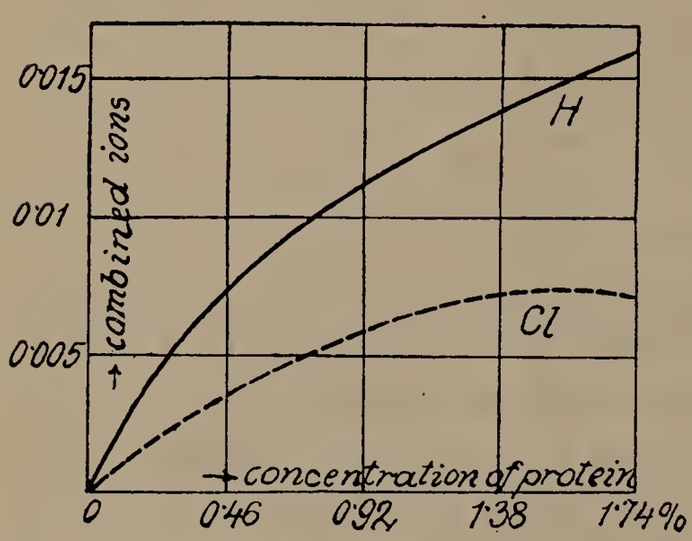


FIG. 12.—Combination with variable protein concentration.

The maximum ionisation of albumin chloride implies a maximum of positively charged albumin particles, and an investigation of the migration of acid albumin by Pauli and Samec * goes to prove this. With constant difference of potential the quantity of albumin moving towards the cathode falls off again when the concentration of acid rises above 0·05 N.

Previous to the more recent electrometric measurements given above, the behaviour of acid albumin on ionisation and the existence of a maximum value for the ionisation were deduced from observations on the viscosity of such mixtures. The work of Pauli and Handovsky † showed that when hydrochloric acid is added to albumin (*e.g.*, a 1 per cent. serum albumin solution), a considerable rise in viscosity occurs. A maximum value is obtained between 0·017 N and 0·02 N acidity, further addition of acid causing a decrease in viscosity (Table 21, Fig. 13).

* Not yet published.
† *Biochem. Zeitsch.*, 1909, **18**, 340.

E. Laqueur and O. Sackur * were the first to observe the increase in viscosity on salt-formation by proteins. Such an increase was found in sodium caseinate, obtained by dissolving casein in caustic soda solution, when, with rising concentration of alkali, a maximum and then a falling off in viscosity was noted. They attributed the increase in viscosity to the free casein ions. W. B. Hardy † found a similar increase in viscosity on dissolving globulin in acids or bases. Laqueur and Sackur concluded that the decrease in viscosity with excess of alkali was due to the effect of the common ion of the sodium hydroxide in repressing the ionisation of the protein salt, as sodium chloride also produces this result. It is easy to show,

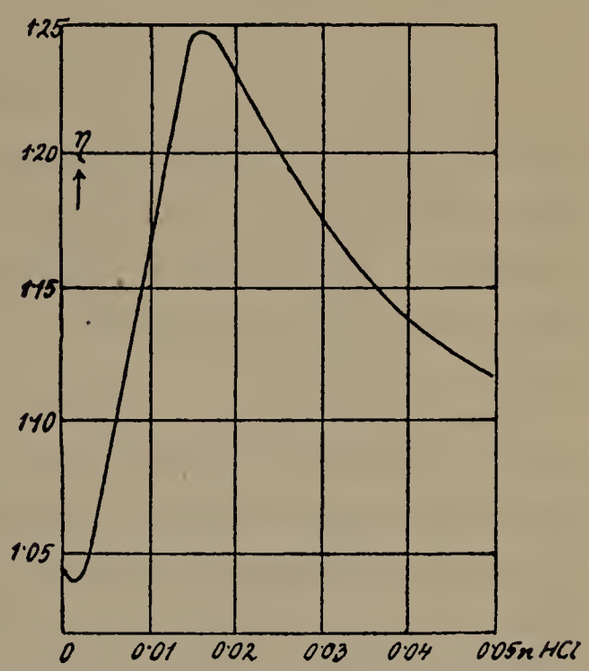


FIG. 13.—Viscosity of albumin in hydrochloric acid.

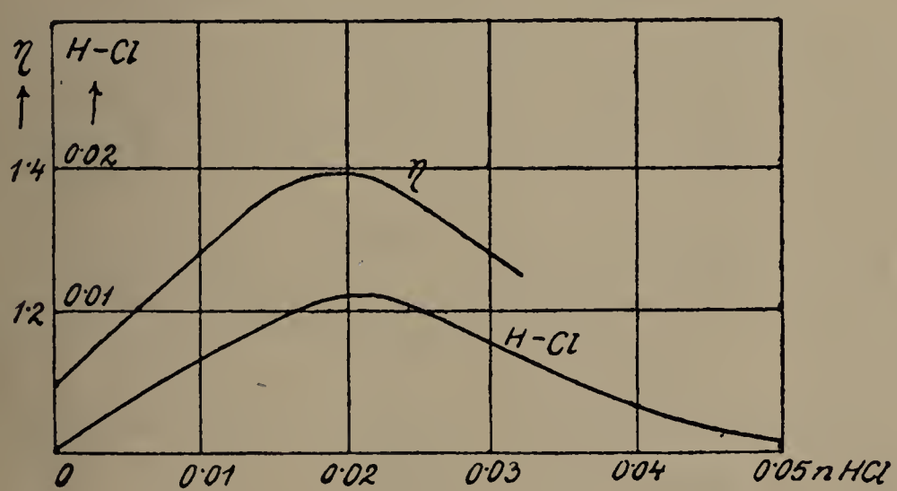


FIG. 14.—Viscosity and difference in ion concentration.

however, that neutral salts of varied cation affect the viscosity of sodium caseinate in the same way. It is, in fact, an effect produced by salts in general, as we shall see later, and thus the

* *Beitr. z. Chem. Physiol. u. Path.*, 1903, **3**, 193.
† *J. Physiol.*, 1905, **33**, 251.

Table 21 (Fig. 13).

Concentration of HCl.	0.0	0·005	0·01	0·015	0·017	0·02	0·03	0·04	0·05 N
η	1·041	1·083	1·166	1·243	1·243	1·232	1·165	1·136	1·121

observations of Laqueur and Sackur on the effect of salts lose their validity as an explanation of the alkalinity-viscosity curve of casein. Nevertheless, their view, that in excess of alkali the ionisation of the caseinate is suppressed, has proved to be correct.

Pauli and his co-workers have explained the course of the viscosity curve of hydrochloric acid and albumin by the same ionic conceptions as those employed by Laqueur and Sackur. The work of Manabe and Matula has finally settled the parallelism between the viscosity curve and the ionisation curve as determined by the electrometric method (Table 21A, Fig. 14).

Table 21a.

Concentration of HCl.	Ox-serum (1·61%).		Ox-serum (0·8%).	
	H· – Cl′.	t/t_0.	H· – Cl′.	t/t_0.
0	—	1·077	—	1·035
3×10^{-3}	$1·83 \times 10^{-3}$	—	$5·65 \times 10^{-4}$	—
4×10^{-3}	—	—	—	1·079
5×10^{-3}	$3·15 \times 10^{-3}$	—	$1·51 \times 10^{-3}$	—
6×10^{-3}	—	—	—	1·133
7×10^{-3}	$4·64 \times 10^{-3}$	—	$3·01 \times 10^{-3}$	—
1×10^{-2}	$5·81 \times 10^{-3}$	—	$5·03 \times 10^{-2}$	1·204
$1·2 \times 10^{-2}$	—	—	$3·94 \times 10^{-3}$	1·212
$1·5 \times 10^{-2}$	$6·72 \times 10^{-3}$	1·343	—	—
$1·6 \times 10^{-2}$	—	—	—	1·204
$1·7 \times 10^{-2}$	—	1·383	$2·94 \times 10^{-3}$	—
2×10^{-2}	$1·01 \times 10^{-2}$	1·391	$2·74 \times 10^{-3}$	1·186
$2·2 \times 10^{-2}$	—	1·385	—	—
3×10^{-2}	$6·06 \times 10^{-3}$	1·289	—	—
5×10^{-2}	$8·5 \times 10^{-4}$	—	$6·0 \times 10^{-4}$	1·107

We give next a very complete series of careful measurements of the viscosity of horse-serum albumin (free from salts) made by Pauli and R. Wagner,* which include the initial decrease.

* *Biochem. Zeitsch.*, 1910, **27**, 297.

They accordingly embrace the two change points *c* and *d* of acid albumin referred to in the preceding chapter.

Table 22.

Albumin (1 per cent.).

N.HCl.	Relative viscosity.	N.HCl.	Relative viscosity.
0·0	1·088	0·02	1·487 *
0·001	1·086	0·024	1·451
0·0016	1·084	0·03	1·426
0·002	1·084 *	0·04	1·353
0·0024	1·086	0·05	1·284
0·004	1·102	0·06	1·268
0·006	1·128	0·08	1·228
0·01	1·322	0·1	1·199
0·016	1·402	—	—

Glutin gives similar results to those obtained in viscosity measurements on albumin, and is particularly useful in determining the effect of temperature on viscosity. With a falling temperature a disproportionate increase in viscosity takes place in the region of the maximum value, whereas the concentration of acid which gives the maximum viscosity is practically independent of temperature for a given content of glutin. As might be expected from the electrometric results, the maximum viscosity moves in the direction of greater acidity when the concentration of glutin is raised. All these relations can be seen from the results of Pauli and O. Fálek * given in Table 23.

Table 23.

Concentration of HCl.	Relative viscosity 0·3% glutin.			1% glutin. 35°.
	30°.	35°.	40°.	
0	1·1236	1·1069	1·0934	1·5540
0·002 N	1·3018	—	1·1964	1·6644
0·0025	—	1·2970	—	—
0·003	1·3905	1·3423	1·2938	1·8598
0·004	1·4077	1·3743	1·3463	1·9563
0·005	1·4302 *	1·3850 *	1·3683 *	2·0988
0·01	1·3291	1·2889	1·2821	2·7632 *
0·02	—	1·2202	—	2·5149

* *Biochem. Zeitsch.*, 1912, **47**, 269.

In connection with the above viscometric measurements, C. Schorr * has made the important observation that the quantity of the precipitate produced by alcohol in acid albumin solution passes through a minimum value when increasing quantities of acid are added. The point of minimum precipitate coincides with the maxima of viscosity and of ionisation, and still larger quantities of acid cause the quantity precipitated to increase again. The following table gives the results of work on a 1·1 per cent. ox-serum. The mixture of albumin and acid was precipitated by the addition of ten times its volume of alcohol.

Table 24.

Concentration (× N).	Hydrochloric acid.	Trichloracetic acid.	Acetic acid.
0·001	+ + +	+ + +	+ + +
0·002	± + +	+ + +	+ + +
0·003	± ±	± + +	± + +
0·005	±	+	± + +
0·01	— ±	±	+ +
0·02	—	—	± +
0·025	—	—	± ±
0·04	— ±	—	± ±
0·05	∓	—	+
0·1	±	±	∓
0·2	+	± ±	± —
0·3	± ±	+ + +	— —
0·5	—	+ + +	— —

Measurements of the depression of the freezing point, due to Bugarsky and Liebermann,† lead to the result that addition of albumin to solutions of hydrochloric acid causes a decrease in the lowering of freezing point, showing that the molecular concentration has become less. The molecules of the albumin salt formed on combination of the albumin with the acid cause a diminution in the total number of molecules of albumin *plus* acid. Further, by working out the results of these authors it is clear that when increasing quantities of albumin are added to a constant quantity of acid, the depression caused per gram of albumin first of all increases and then falls off

* *Biochem. Zeitsch.*, 1911, **37**, 424.
† *Loc. cit.*

again. As we can now see, this is due to the appearance of free Cl′ ions in high concentrations of albumin, which take part in the lowering of the freezing point. With lower albumin content the excess of acid present represses the ionisation of the albumin chloride. The actual results are shown in Table 25, in which Δ is the observed depression, D the depression calculated from the sum of acid and albumin molecules, and $\frac{D - \Delta}{g}$ the difference produced per gram of albumin.

Table 25.

(*g = grams egg albumin ; 0·05 N HCl in all cases.*)

g.	Δ.	D − Δ.	$\frac{D - \Delta}{g}$.
0	0·186	0	—
0·2	0·184	0·004	—
0·4	0·181	0·007	17·5
0·8	0·172	0·02	25·0
1·6	0·146	0·049	30·0
3·2	0·101	0·101	31·8
6·4	0·087	0·121	19·0

We can now summarise the information supplied by these various researches on the behaviour of albumin and glutin in the presence of hydrochloric acid as follows. On adding acid to a given weight of protein, combination occurs to an extent which increases with increase of acid up to a maximum. Thus, for 1 gm. serum albumin the maximum combination is with $1{\cdot}66 \times 10^{-3}$ gm. mol. HCl or 60·59 mg. HCl. ; for 1 gm. glutin the value is $1{\cdot}5 \times 10^{-3}$ gm. mol. HCl or 54 mg. HCl. When combination occurs, the hydrogen of the acid forms with the albumin an electro-positive albumin ion, while the chlorine ions largely remain unattached. With excess of acid, the ionisation of the protein chloride is suppressed, as is shown by a rapid increase in the combination of the chlorine ions with the protein, and also by a decrease in the quantity of albumin transported in the electric field. The hydration of the protein particles, moreover, increases as the combination with acid

and the consequent ionisation proceeds, as is shown by the increase in viscosity, which, however, decreases once again when excess of acid has reduced the ionisation, and thus increased the number of neutral particles. The precipitation by alcohol also is reduced to practically nil as the ionisation of the acid protein increases, but reappears again when the ionisation is reduced.

The difference in properties between ionised and neutral albumin, which has already been sketched in the previous chapter, and to which we shall return later, is reflected in the behaviour of albumin chloride. But, whereas the behaviour of albumin in the iso-electric region (in the presence of buffer solutions) is a question of the existence of ionic and of neutral particles of natural albumin (or of the polyamino-acids of E. Fischer), we have here to deal with the ionic and neutral particles of the albumin salts formed when the protein combines with acids. The structure of these salts must now be discussed, and first of all the salts formed with different acids must be considered.

The ideas so far presented have been based on the salts of proteins with hydrochloric acid, on account of the ease with which the behaviour on dissociation can be investigated by measurements of the $H^{\cdot}$ and Cl' ion concentrations. Corresponding measurements with bromide and iodide have also been possible, but no remarkable differences between these salts and the chloride have appeared. On the other hand, electrometric measurements with polyvalent anions such as SO_4'' and PO_4''' fail because the accuracy of such measurements decreases considerably with increasing valency of the anion. It is insufficient even for SO_4, according to experiments made by Pauli and Hirschfeld.* Hence, it is necessary to rely entirely on measurements of $H^{\cdot}$ ion concentrations when investigating the combination of proteins with acids other than the halogen hydracids. The fraction of the acid which combines with the protein can therefore only be determined in this way in the case of strong acids or of weak acids of known dissociation constant. These methods depend on the assumption which applies to all salts, that the protein salts are con-

* *Biochem. Zeitsch.*, 1914, **62**, 245.

siderably dissociated and to an extent independent of the strength of the acid.

According to Arrhenius, if a strong acid is partly neutralised by an alkali (by a protein in the case we are considering), the portion of the acid which remains free has the same degree of dissociation as that of the original acid in the same volume. Thus, a mixture containing 0·05 N H_2SO_4 and 0·05 N Na_2SO_4 is dissociated to the same extent as 0·1 N H_2SO_4. If α is the degree of dissociation of an acid in concentration n, and after partial neutralisation without alteration in volume the concentration of free acid is n', the portion of the acid dissociated is $n'\alpha$. A measurement of the hydrion concentration gives this value $C_H = n'\alpha$, and thus the combined (or neutralised) part of the acid, $n - n' = \frac{n - C_H}{\alpha}$.

In this way it is possible to obtain a value for the combination of albumin with strong acids from a knowledge of the original concentration of acid, n, the corresponding degree of dissociation, α, and the hydrion concentration, C_H, measured after addition of the protein. Thus Pauli and Hirschfeld found that the quantity of sulphuric acid which entered into combination with albumin and glutin was nearly the same as for hydrochloric acid. It is, however, rather less than the latter in high concentrations.

Table 26 (Fig. 15).

Horse-serum Albumin (1·26 per cent.).

Concentration of acid.	Hydrochloric acid.		Sulphuric acid.	
	C_H.	Combined per litre.	C_H.	Combined per litre.
0·001 N	—	—	$1·02 \times 10^{-5}$ N	$3·89 \times 10^{-4}$ N
0·002	$1·77 \times 10^{-5}$ N	$1·982 \times 10^{-3}$ N	—	—
0·003	$6·68 \times 10^{-5}$	$2·93 \times 10^{-3}$	—	—
0·005	$2·15 \times 10^{-4}$	$4·779 \times 10^{-3}$	$1·77 \times 10^{-4}$	$4·792 \times 10^{-3}$
0·007	—	—	$2·85 \times 10^{-4}$	$6·655 \times 10^{-3}$
0·01	—	—	$8·31 \times 10^{-4}$	$8·95 \times 10^{-3}$
0·02	$3·24 \times 10^{-3}$	$1·661 \times 10^{-2}$	$3·4 \times 10^{-3}$	$1·539 \times 10^{-2}$
0·025	$5·7 \times 10^{-3}$	$1·902 \times 10^{-2}$	$5·32 \times 10^{-3}$	$1·761 \times 10^{-2}$
0·04	$1·81 \times 10^{-2}$	$2·08 \times 10^{-2}$	$1·48 \times 10^{-2}$	$1·82 \times 10^{-2}$
0·05	$2·45 \times 10^{-2}$	$2·39 \times 10^{-2}$	$2·04 \times 10^{-2}$	$1·87 \times 10^{-2}$

The degree of combination of weak acids can be derived from the values of C_H on the basis of the law of mass action. The method of calculation is shown by the following example, as worked out by the above authors for the acetic acid—acetate mixtures which they used.

When an acetate is added to acetic acid the dissociation isotherm of the acid

$$K \cdot C'_{CH_3.COOH} = C'^{2}_{H}$$

is transformed into the expression

$$K \cdot C_{CH_3.COOH} = C_H \cdot (C_H + C_{CH_3COO}) \quad . \quad . \quad . \quad (1)$$

where K is the dissociation constant of acetic acid ($1{\cdot}8 \times 10^{-5}$), C_H the concentration of free hydrions, and C_{CH_3COO} the concentration of the acetate ions which have their origin in the combination with albumin. We can equate the latter value to the hydrogen combined with the albumin, or to the acid combined therewith, as the albumin salt is highly dissociated in the dilute solution. The number of acetate ions arising from the free acid must be equal to C_H. Further, if n is the normality of the total acetic acid employed in the reaction, the following relation holds between it and the undissociated part of the acid C_{CH_3COOH}:

$$C_{CH_3COOH} = n - C_H - C_{CH_3COO}.$$

Substituting in equation 1

$$K(n - C_H - C_{CH_3COO}) = C_H (C_H + C_{CH_3COO}),$$

so that the acid combined with the protein is

$$C_{CH_3COO} = \frac{K(n - C_H) - C_H^2}{K + C_H} \quad . \quad . \quad . \quad . \quad (2)$$

In this way the combination of acetic acid and albumin can be studied, and, indeed, the expression is capable of general application provided the dissociation constant of the acid is known, and the salts formed with albumin are considerably dissociated (see Table 27, Fig. 15).

The measurements of Pauli and Hirschfeld given below show that the combination of albumin with acetic acid falls considerably short of that which occurs with strong acids. This

is, however, only true if acids of the same normal concentration are compared ; if solutions of the same hydrion concentration are compared, then the quantity of the weaker acetic acid which

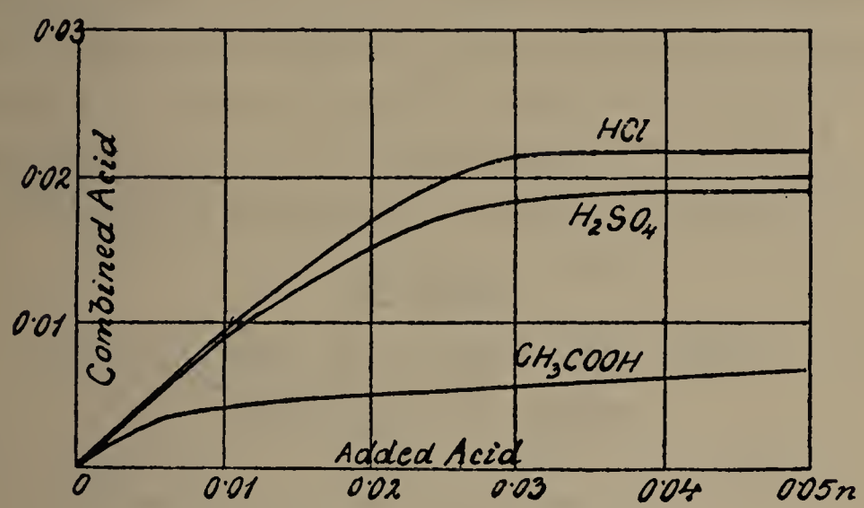


FIG. 15.—Combination with various acids.

combines is notably greater. Thus a 0·2 N acetic acid corresponds to a 0·002 N hydrochloric acid ; but nearly 9×10^{-3} N acid is combined in the former case or more than four times as much as in the hydrochloric acid solution. The same result is met with in the swelling of gelatin in various acids.

Table 27 (Fig. 15).

Horse-serum Albumin (1·26 per cent.) + Acetic Acid.

Concentration of acid.	α of the acetic acid.	H·ion concentration of the acetic acid.	C_H of the mixture.	Combined acid.
0·005 N	0·0569	$2{\cdot}85 \times 10^{-4}$ N	$1{\cdot}31 \times 10^{-5}$ N	$3{\cdot}00 \times 10^{-3}$ N
0·02	0·0296	$5{\cdot}92 \times 10^{-4}$	$4{\cdot}97 \times 10^{-5}$	$5{\cdot}24 \times 10^{-3}$
0·04	0·0211	$8{\cdot}44 \times 10^{-4}$	$1{\cdot}02 \times 10^{-4}$	$6{\cdot}00 \times 10^{-3}$
0·05	0·0184	$9{\cdot}20 \times 10^{-4}$	$1{\cdot}22 \times 10^{-4}$	$6{\cdot}47 \times 10^{-3}$
0·1	0·0131	$1{\cdot}31 \times 10^{-3}$	$2{\cdot}27 \times 10^{-4}$	$7{\cdot}11 \times 10^{-3}$
0·2	0·090	$1{\cdot}8 \times 10^{-3}$	$3{\cdot}91 \times 10^{-4}$	$8{\cdot}6 \times 10^{-3}$
0·4	0·0066	$2{\cdot}64 \times 10^{-3}$	$6{\cdot}88 \times 10^{-4}$	$9{\cdot}47 \times 10^{-3}$
0·5	0·00572	$2{\cdot}86 \times 10^{-3}$	$9{\cdot}79 \times 10^{-4}$	(?) $9{\cdot}84 \times 10^{-3}$

In the work of Manabe and Matula already quoted, in which the relation between combination with acid and concentration of albumin was studied, a relative increase in the extent of combination as the content of albumin decreased was observed.

A similar effect was noticed with acetic acid. Thus in Table 28 the quantity of combined acid in a half and a quarter of the original albumin content is distinctly greater than a half and a quarter of that found in the original concentration. This result is due to the suppression of hydrolysis by the relative excess of acid in the lower concentrations of albumin (see below). It does not represent, however, the maximum combination, which takes place only in considerable excess of acid.

Table 28.

Acetic Acid + Ox-serum Albumin of various Concentrations.

Concentration of acid.	Acid combined with albumin of concentration.		
	1·26%.	0·63%.	0·315%.
0·005 N	$3{\cdot}00 \times 10^{-3}$ N	—	$2{\cdot}206 \times 10^{-3}$ N
0·01	—	$3{\cdot}12 \times 10^{-3}$ N	—
0·05	$6{\cdot}47 \times 10^{-3}$	—	$4{\cdot}96 \times 10^{-3}$
0·1	$7{\cdot}11 \times 10^{-3}$	$6{\cdot}97 \times 10^{-3}$	—

On the other hand, in excess of acid, and when the maximum combination has been attained, the quantity of combined acid

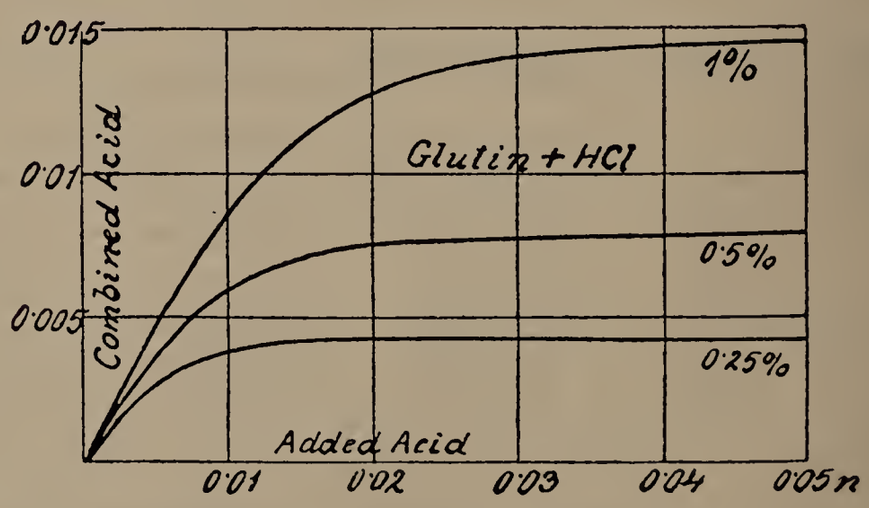


Fig. 16.—Glutin and hydrochloric acid.

is proportional to the albumin content, and consequently this value is specific for the protein concerned. Fig. 16, from the

work of Pauli and Hirschfeld, shows the behaviour of 1·0, 0·5 and 0·25 per cent. glutin very clearly.

The studies of Pauli with H. Handovsky, O. Falek and M. Hirschfeld on the relations of viscosity to the combination of albumin with various acids are particularly interesting.

Table 29.

Relative Viscosities of Ox-serum Albumin with different Acids.

(*Relative viscosity of the serum albumin* = 1·041.)

Concentration of acid.	Hydrochloric acid.	Sulphuric acid.	Trichloracetic acid.	Citric acid.	Acetic acid.
0·01 N	1·166	1·061	1·073	1·044	1·046
0·02	1·232	1·061	1·059	1·066	1·052
0·03	1·165	1·060	1·053	1·100	1·066
0·04	1·136	1·064	1·056	1·111	1·075
0·05	1·121	1·066	1·060	1·141	1·091

Let us consider first the viscosity curves of albumin chloride and acetate. The former rises steeply to a maximum, while the latter shows a gentle slope upwards. The difference is satisfactorily explained by the lower strength of the acetic acid, which leads to a less extensive formation of albumin ions, but not to a repression of the dissociation of the strongly ionised albumin acetate. This conception, however, fails to account for the behaviour of the strong trichloracetic and sulphuric acids, which display a much smaller increase in viscosity than either acetic acid or even much weaker acids, such as monochloracetic, oxalic and citric acids, which give a larger increase in the viscosity of the protein. As the electrometric measurements give no basis for the supposition that differences in degree of combination occur with equally strong acids, the variation in viscosity in this case can only be due to differences in hydration caused in some other way. Such differences in hydration can be accounted for either by varied ionisation of the individual protein salts, which when less ionised would be less viscous, or by the difference in structure of the positive albumin ions of

different protein salts, in which case variations in hydration of the ions and of the neutral particles can have a combined effect. The latter alternative can be regarded at once as improbable, and, in analogy with typical metallic salts, we may assume the identity of the albumin ions of the protein salts in the same way that the same cations are found to be given by the salts of a metal with different acids. Accurate electrometric measurements on albumin chloride show, however, that a small portion of the acid combines with the protein in a manner which appears to be molecular, both the ions taking part. Considerable differences in the properties of the albumin ions of different protein salts may be caused by this portion of the acid; and that small quantities of substances can effect considerable changes in the properties of the ions of acid albumin is shown by investigations of the combination of acid albumin and neutral salts (see later). The molecular reaction of the uncombined part of the acid must also be considered, and it is probably no mere accident that the marked anomaly of sulphuric and trichloracetic acids is coincident with their considerable power of dehydration. As, however, the relations in ionisation of the various protein salts, as determined by free anions, are at the moment far from clear, a decision as to the origin of the differing hydration of these salts must be sought in other ways. In this respect the researches of Pauli and Samec * on the optical rotation of protein salts provide some useful suggestions. These authors found that even in low concentrations both acids and bases give rise to an increase in the optical rotation of albumin. The table and

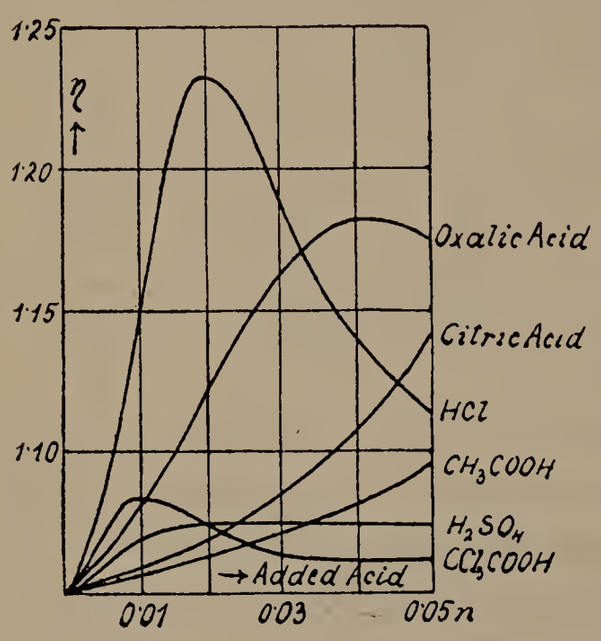


FIG. 17.—Viscosity curves with various acids.

* *Biochem. Zeitsch.*, 1914, **59**, 470.

figure following give the results of investigations on serum albumin.

Table 30.

(*α° of the pure albumin* = 1·02° ;
ox-serum albumin concentration = 1·07 *per cent.*)

Concentration of acid.	HCl.	H_2SO_4.	COOH. | COOH.	CH_3 . COOH.	CH_2Cl. | COOH.	CCl_3. | COOH.
0·005 N	1·13°	1·07°	1·06°	1·025°	1·07°	1·04°
0·01	1·25	1·09	1·12	1·03	1·13	1·05
0·02	1·38	1·12	1·18	1·04	1·18	1·07
0·04	1·39	1·17	1·30	1·30	1·20	1·08
0·05	1·39	1·19	1·34	1·08	1·22	1·08

These and other similar experiments show that for an increase in rotation it is not the strength of the acid that is decisive. Thus, oxalic acid is more effective than the much stronger trichloracetic acid or sulphuric acid, and acids as different in strength as trichloracetic and acetic acids have much the same effect on the optical rotation. In this respect there is a notable agreement between the curves for viscosity and for optical rotation of the protein salts of the various acids.

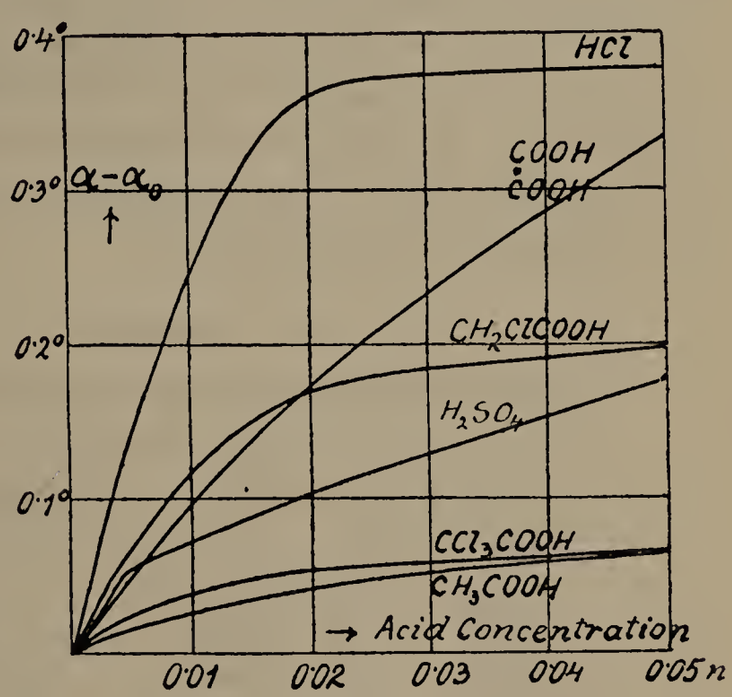


Fig. 18.—Rotation curves with various acids.

On the other hand, the optical rotation fails to give the maximum value and subsequent decrease that is so characteristic of the viscosity, *e.g.*, of hydrochloric acid and proteins. The rotation tends rather to remain constant after reaching the maximum. It is thus the general formation of salts of albumin, and not

their ionisation, which is important in determining the optical rotation. We must therefore seek the explanation of the differences in rotation of the protein salts in constitutive differences of the protein portion of these salts rather than in variations in ionisation with a common anion. It is extremely probable that the widespread agreement between rotation and viscosity in the series of albumin salts with various acids is due to the same or, at least, similar constitutive differences exerting their influence on the viscosity of the acid proteins.

The very recent work of M. Adolf and E. Spiegel * on an acid-albumin made by boiling dialysed ox-serum albumin with dilute hydrochloric acid has shown with certainty that, when the precipitated acid albumin is dissolved in increasing quantities of hydrochloric acid, the rotation passes through a maximum. Unlike natural albumin, however, of which the rotation remains at the maximum level on further addition of acid, with this material the value decreases in more acid solutions until it finally sinks to the original value.

Table 31 gives some of these results. The values of the viscosity for increasing concentrations of acid are added for comparison, showing the characteristic course of the curve with a sharp peak at the maximum point due to repression of the ionisation in excess of acid.

Table 31.

(*Final concentration of acid-albumin,* 0·62 *per cent.*)

Concentration of hydrochloric acid.	Rotation $\alpha°$.	Concentration of hydrochloric acid.	Relative viscosity.
0·008 N	— 0·29°	0·008 N	1·154
0·028	— 0·33	0·013	1·196
0·058	— 0·49	0·018	1·158
0·079	— 0·33	0·028	1·135
0·108	— 0·29	0·048	1·103
—	—	0·058	1·103

If, on the one hand, the viscosity (Table 31) and the extent of combination of acid-albumin (Table 39) are compared with

* *Biochem. Zeitsch.*, 1920, **104,** 175.

the variation in optical activity with increasing addition of acid on the other hand, it is clear that not only the viscosity maximum, but also the combination with the greatest quantity of the acid employed and the ionisation maximum are coincident. The peak of the rotation curve does not coincide but lies rather on the part representing the general decrease in the viscosity. It is not, apparently, a difference in the rotation of the ions and the neutral molecules of the protein salts, but rather an effect of higher acid concentration on the constitution of the acid-albumin that here determines the characteristic course of the curve for rotation.

C. Schorr's * work on the precipitation by alcohol of the acid albumins of different acids gives the same interesting difference, which, for hydrochloric acid and acetic acid, lies entirely in the comparison with the viscosity curves in the two cases. In the first instance the maximum of viscosity corresponds to one of precipitation; in the second case the increase in the alcohol precipitate only appears in higher concentrations of the acid. With albumin sulphate low viscosity corresponds well with greater precipitation by alcohol, so much so that to show the variation in precipitation—decrease and subsequent increase with continuously increasing acid concentration—it is necessary to employ a 45 per cent. alcohol in place of the 96 per cent. alcohol used in the work with hydrochloric acid. The ionisation effect is then clearly seen. Albumin trichloracetate falls quite outside any connection between viscosity and alcohol precipitation, as it is entirely precipitated in analogy with albumin chloride. The viscosity in this case indicates a great difference in the hydration (Table 24). Here the strength of the acid is clearly expressed in a corresponding difference of ionisation of the albumin salt, as shown by the effect of addition of alcohol. In general, the precipitation by alcohol, as shown by the researches which have been quoted, is an indicator of the dissociative properties of the salt examined in this way.

A comparison of the viscosity values in concentration of acid of 0·05 N and the precipitation of the albumin by acids is

* *Biochem. Zeitsch.*, 1911, **37**, 424.

particularly instructive. It is well known that in high acid concentrations albumin is precipitated with formation of a coagulum, which is not dispersed again on dilution. If this irreversible change is regarded as a secondary effect, one can reasonably suppose that this precipitation is only effective on the neutral particles, for only such can be deposited out of a solution.

It can further be supposed that neutral particles are more soluble the more they are hydrated. The viscosity of the acid proteins after the maximum has been passed is a measure of the number of neutral particles, and therefore of their hydration; consequently a relation between viscosity and precipitation by acids is to be expected in the sense that low viscosity and low precipitation will be coincident. This has actually been found by Pauli and R. Wagner,* who measured the concentration of various acids required to precipitate a serum albumin free from salts (Table 32).

Table 32.

Acid 0·05 N.	Relative viscosity (25°), 1 % albumin + 0·05 N acid.	Concentration (of the acid required	H· ion concentration to precipitate at 18°).
CCl_3COOH	1·0603	0·04 N	0·0356 N
CCl_2HCOOH	—	0·01	0·0981
H_2SO_4	1·0656	0·2	0·1163
CH_3COOH	1·0906	11·2	0·1411
HNO_3	—	0·2	0·1813
HCl	1·1206	0·4	0·3517
$CH_2ClCOOH$	1·2156	1·5	0·4108

The agreement in this table is very satisfactory, particularly when one considers that the final products of the acid precipitation exhibit considerable differences in properties (*e.g.*, solubility in excess of acid). The reaction of nitric acid with albumin does not permit of viscosity measurements at 25° when more than 0·01 N acid is present. Even better agreement is obtained when the series of hydrion concentrations, corresponding to the

* "*Anzeiger Akad. Wissensch. Wien.*" 1910. No. IX. *Biochem. Zeitsch.*, 1920, **104**, 190.

concentration of acid which produces precipitation, is compared with the viscosity. It increases with increasing viscosity (*i.e.*, hydration). These results become comprehensible if the precipitation of the acid albumin by excess of acid is considered as the action of the common ion of the acid, in the same way that common salt is precipitated by hydrochloric acid. In this way an excess of dissociated acid is required to precipitate the acid protein, which is the greater when the number of neutral particles is less, and when they are heavily hydrated. This conception makes the precipitation of albumin in high acid concentrations intelligible as a displacement of the albumin salt from solution. The completeness of the precipitation, the varied behaviour with excess of acid, etc., are, however, secondary effects which depend on the acid used, and will not be further entered into at this point.

CHAPTER VI

SALTS OF ALBUMIN AND ACIDS (*continued*)

THE question of the hydrolytic dissociation of the albumin salts is very important for a complete understanding of these substances. It is well known that the salts of weak acids or of weak bases have the property of reacting in aqueous solution with formation of notable quantities of free acid and base.

In accordance with the equation :

$$BS + H_2O \rightleftharpoons SH + BOH \quad . \quad . \quad . \quad (1)$$

if the acid is the stronger the hydrion concentration is increased owing to the dissociation :

$$SH \rightleftharpoons S' + H^{\cdot},$$

or, if the base is stronger than the acid, the greater ionisation of the former gives rise to more OH′ ions. In the presence of excess of one of the products of hydrolysis, the hydrolytic dissociation is repressed in accordance with the law of mass action, so that in equation 1 the reaction is reversed in the direction of the upper arrow, *i.e.* from right to left. The fact that the combination of albumin with acids or alkalis increases with excess of acid or of alkali lends support to the view that the protein functions as a weak base or weak acid under these conditions, and so the addition of free acid or base respectively opposes the hydrolytic dissociation of the albumin salt. This view, notwithstanding the lack of convincing quantitative measurements, held the field until recently (for literature see Cohnheim, " Chemie der Eiweisskörper," 3e Auflage).

Robertson (*loc. cit.*), after his work on casein, opposed this conception and decried altogether the hydrolytic dissociation of the albumin salts. As a matter of fact, the behaviour throughout is not nearly as simple as would appear from the usual descriptions of it, and on account of the importance of the matter it will now be further considered.

Experience shows that albumin mixed with acid at low concentration (up to 0·05 N), if it is not warmed above room temperature and not kept for more than a few days, can be freed from the acid by dialysis without any change in its original properties.* On the basis of what we know of albumin salts it can be stated without a doubt that here the dialysis operates by hydrolytic elimination of the acid at the bounding membrane and subsequent exosmosis of the acid. This experience can, moreover, be expressed and confirmed experimentally by saying that the curve of acid combination (Table 18, Fig. 10) for decreasing acid concentration coincides point for point with that obtained for increasing concentrations of acid. This indicates a homodrome reversible change of state. Let us now consider rather more closely the work of Pauli and Hirschfeld (*loc. cit.*, p. 68) as shown in Table 33 and Fig. 19.

Table 33 (Fig. 19).

Horse-serum Albumin (1·26 per cent.).

Concentration of acid.	HCl combined.	H_2SO_4 combined.	CH_3COOH combined.
0·002	$1·90 \times 10^{-3}$	—	—
0·005	$4·78 \times 10^{-3}$	$4·79 \times 10^{-3}$	$3·0 \times 10^{-3}$
0·01	—	$8·95 \times 10^{-3}$	—
0·02	$1·66 \times 10^{-2}$	$1·54 \times 10^{-2}$	$5·24 \times 10^{-3}$
0·04	$2·08 \times 10^{-2}$	$1·82 \times 10^{-2}$	$6·00 \times 10^{-3}$
0·05	—	$1·87 \times 10^{-2}$	$6·47 \times 10^{-3}$
0·1	—	—	$7·11 \times 10^{-3}$
0·2	—	—	$8·6 \times 10^{-3}$
0·4	—	—	$9·47 \times 10^{-3}$

The 1·26 per cent. serum albumin employed is capable of combining with a maximum quantity of $2·1 \times 10^{-2}$ N acid per litre. This limiting value, however, only occurs in a considerable excess of acid (0·05 N). If, now, $2·1 \times 10^{-2}$ N acid is added to albumin, instead of AC, only AB (Fig. 19) represents the combined acid so that the degree of hydrolysis, that is, the fraction of the total concentration which is free, is BC/AC. In

* Such an albumin is called *natural* or *genuine*; whereas one of which the properties (*e.g.*, solubility) have been altered by irreversible transformation without complete decomposition is termed *denatured*.

a similar way the degree of hydrolysis with increasing addition of acid can be read off within OA, as the corresponding quantity of acid which remains free is included in the area OBC. Further, the course of the curve OB shows that the degree of hydrolysis steadily increases up to the value of the combined acid represented by AB. Beyond that point the excess of acid rapidly increases, and the hydrolysis is suppressed, as is easily seen by the form of the area BCD. At D the hydrolysis of the protein salt is practically nil. The tabular statement which follows, giving in the usual way the mean hydrolysis of the serum albumin used with increasing acid concentration, demonstrates the same variation.

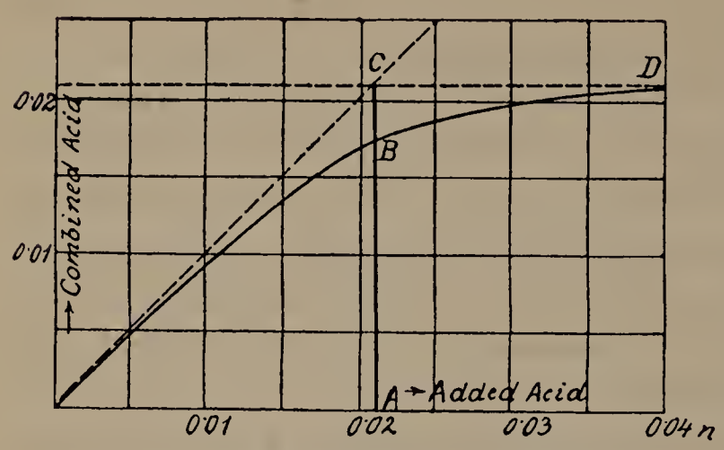


FIG. 19.—Hydrolysis of acid albumin.

Table 34.

N.HCl.	Hydrolysis per cent.	K_b (v = 1000).
0·005	5·0	$2·36 \times 10^{-8}$
0·01	7·5	$1·03 \times 10^{-9}$
0·024	18·0	$1·57 \times 10^{-10}$
0·025	8·57	—
0·03	4·76	—
0·04	1·1	—

Our observations, therefore, support the view that the combination with acid increases proportionally with the content of albumin. The value of maximum combination of $2·1 \times 10^{-2}$ N HCl per litre for 1·26 per cent albumin gives for a concentration of 1 per cent. albumin the value $1·66 \times 10^{-2}$ N HCl. K. Manabe and J. Matula * obtained as a maximum value $1·8 \times 10^{-2}$ N. acid for a 1·09 per cent. solution of serum albumin, which is equal to $1·65 \times 10^{-2}$ N HCl for a 1 per cent. solution.

* *Biochem. Zeitsch.*, 1913, **52**, 385.

Pauli and Hirschfeld found for a 1 per cent, solution of the purest serum albumin the value $1{\cdot}66 \times 10^{-2}$ N HCl. This 1 per cent albumin gave, in a solution of hydrochloric acid of $1{\cdot}66 \times 10^{-2}$ N strength, a hydrolysis of 18·7 per cent.

The proportionality between optimum combination with acid and content of protein is also shown in an experiment of Pauli and Hirschfeld on glutin (see Fig. 16). In a solution which contains 1 per cent. glutin in acid of concentration $1{\cdot}5 \times 10^{-2}$ N HCl, (the maximum quantity combined at that concentration of the protein), the hydrolysis is 25 per cent. We have now every evidence for the view, as opposed to that developed by Robert-

Table 35.

	Dilution.	Content of		Hydrolysis per cent.	Hydrolysis calculated from $\sqrt{v}$.
		Protein per cent.	HCl.		
Albumin	—	1·26	0·022 N	18·7	—
	1 : 1	0·63	0·011	30·4	—
	1 : 3	0·315	0·00505	38·4	37·4
Glutin	—	1·0	0·015 N	25·00	—
	1 : 1	0·5	0·0075	29·00	—
	1 : 3	0·25	0·00375	40·00	50·0

son, that albumin reacts as a polyacid base. We know from the electrometric measurements which have been quoted and which are typical, that the anion of the added acid gives a dissociated salt, and we must conclude, on the other hand, from the extent of the combination with acid, that several molecules of acid react with one molecule of albumin. For, as the maximum acid combined in 1 per cent. (*i.e.*, 10 gm. per litre) of albumin is $1{\cdot}66 \times 10^{-2}$ N HCl, so for 1 gm. per litre the acid concentration would be $1{\cdot}66 \times 10^{-3}$ N ; and hence, if it is supposed that only one molecule of acid combines with each molecule of albumin, the molecular weight of the latter works out at less than 600. If, however, the albumin reacts as a polyacid base, forming salts with polyvalent ions, for which there is manifold proof, then the behaviour on hydrolytic dissociation becomes intelligible.

Such a compound would first react with acids with its strongest basic valency, and, when that was saturated, the weaker ones would begin to come into play; the hydrolysis also would be very small in low acid concentrations, and would rise to a maximum with increasing addition of acid.

The equilibrium of hydrolysis shows that hydrolytic dissociation increases with dilution. The table on p. 83 shows such an increase in hydrolysis of the protein salt when it is subjected to increasing dilution.

Having thus found the degree of hydrolysis we have a way of determining the mean * basic dissociation constant K_b of the albumin in the protein salt in question. The expression for the hydrolysis constant is:

$$K = \frac{K_{salt} \times K_{H_2O}}{K_{acid} \times K_{base}}$$

and in the case of the salt of a weak base and a strong acid the dissociation constants of the salt and of the acid are practically the same, hence

$$K = \frac{K_{H_2O}}{K_B}$$

If x is the degree of hydrolysis, then $1 - x$ is the non-hydrolysed fraction, and the corresponding concentrations in a volume v are $\frac{x}{v}$ and $\frac{1 - x}{v}$. Accordingly, the equilibrium equation of hydrolysis is

$$K \cdot \frac{1 - x}{v} = \frac{x}{v} \cdot \frac{x}{v}$$

or

$$K = \frac{K_{H_2O}}{K_B} = \frac{x^2}{(1 - x) \cdot v}$$

and hence

$$K_B = \frac{K_{H_2O}\,(1 - x)\,v}{x^2} \quad . \quad . \quad . \quad . \quad . \quad (1)$$

* Albumin as a polyvalent base must exhibit a corresponding number of basic dissociation constants. The values of these constants decrease rapidly from that of the first, but it is not possible at present to determine the separate values. The mean value of K_B for an albumin in its combination with strong acid may be defined as that constant which a *univalent* base would have which displays in like circumstances the same hydrolytic dissociation as the collective available basic valencies of the reacting protein.

At 18° the dissociation constant of water $K_{H_2O} = 0{\cdot}62 \times 10^{-14}$, and if we vary the molecular weight of albumin between the limits 1,000 and 10,000, for a 1 per cent. solution, v will vary correspondingly between 100 and 1,000. The mean basic dissociation constant K_B of the albumin, calculated on a degree of hydrolysis of 0·18 from equation 1, gives the values $1{\cdot}57 \times 10^{-11}$ and $1{\cdot}57 \times 10^{-10}$. Of these two figures the latter probably approaches more nearly to the true value as the molecular weight of the protein is nearer 10,000. The mean value of K_B thus obtained corresponds quite well in its order of magnitude with what we should expect for a high polypeptide, for the value increases from $2{\cdot}3$—$5{\cdot}1 \times 10^{-12}$ for a monoamino-acid (K. Winkelblech) to 2—3×10^{-11} for the simplest dipeptide (H. Euler).

In the formation of albumin salts with acids, the basic valencies of the amino-group operate first according to the equation previously proposed by W. B. Hardy and Wo. Pauli :

$$R\begin{cases} NH_2 \\ COOH \end{cases} + HCl \longrightarrow R\begin{cases} NH_2HCl \\ COOH \end{cases}.$$

The more recently propounded views of Robertson have failed to hold the field in spite of many valuable suggestions, such as that of the *rôle* of peptide linkage, or the possibility of reaction in the enol-form, as no practical proof can be given. On the other hand, it was necessary to decide the question whether the terminal amino-groups, particularly in the diamino-acids, lysin and arginin, are the sole places of combination with acids. This point can be tested by the desamino-proteins which have been deprived of these amino-groups. When acted on by nitrous acid (sodium nitrite + glacial acetic acid) the amino-groups react according to the equation :—

$$\begin{matrix} CH_2NH_2 \\ | \\ COOH \end{matrix} + HNO_2 \longrightarrow \begin{matrix} CH_2OH \\ | \\ COOH \end{matrix} + N_2 + H_2O,$$

a reaction which has also been successfully applied for the determination of amino-nitrogen in proteins. Of the desamino-proteins the most useful for the study of physico-chemical

behaviour is desamino-glutin, which is easily soluble in water (L. Blasel and J. Matula).* With the removal of amino-groups glutin loses completely its characteristic property of gelatinisation. It gives a clear solution of distinctly acid character, as might be expected. It reddens litmus and in 0·75 per cent. solution gives a hydrion concentration of $1{\cdot}79 \times 10^{-5}$ N.

It is remarkable to note, however, that desamino-glutin still shows a considerable power of combining with acids.

Table 36.

0·75 per cent. Desamino-glutin + HCl.

N HCl.	C_H.	Combined acid.
0·005	$8{\cdot}93 \times 10^{-4}$	$4{\cdot}09 \times 10^{-3}$
0·01	$3{\cdot}44 \times 10^{-3}$	$6{\cdot}44 \times 10^{-3}$
0·02	$1{\cdot}14 \times 10^{-2}$	$8{\cdot}11 \times 10^{-3}$
0·03	$2{\cdot}13 \times 10^{-2}$	$7{\cdot}63 \times 10^{-3}$
0·05	$3{\cdot}90 \times 10^{-2}$	$8{\cdot}50 \times 10^{-3}$

Whereas we can estimate the maximum combining capacity of a 0·75 per cent. desamino-glutin solution as 9×10^{-3} N HCl per litre, a glutin solution of equal concentration takes up on the average $1{\cdot}15 \times 10^{-2}$ N HCl. Thus, after removal of amino-groups, about 78 per cent. of the power of combining with acids remains. The chlorion of the combined acid is at first completely dissociated (see Fig. 20) up to an acid concentration of 0·015 N HCl, and then in excess of acid its ionisation is suppressed. Hence desamino-glutin also forms a salt ionised like a typical chloride, and, further, a consideration of the

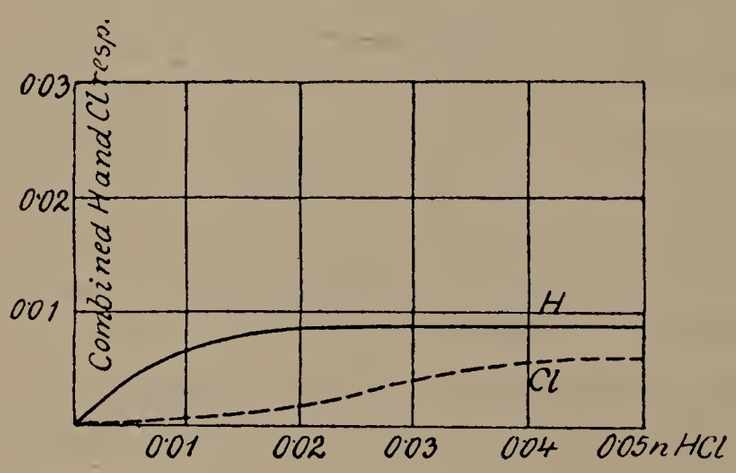
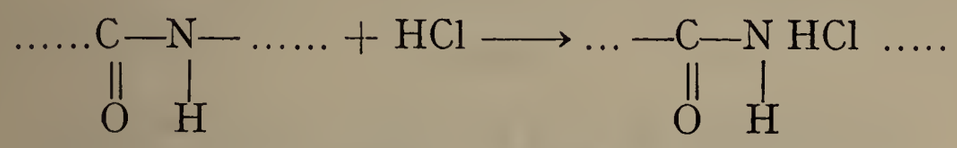


FIG. 20.—Desamino-glutin and hydrochloric acid.

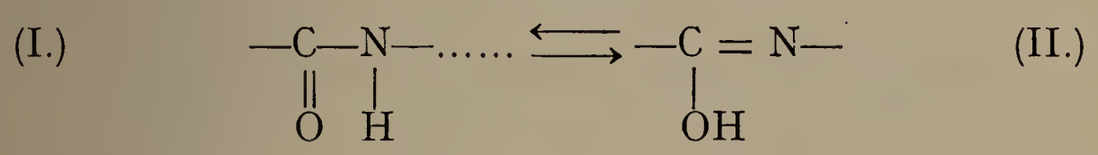
* *Biochem. Zeitsch.*, 1914, **58**, 417.

quantity of acid combined shows that several molecules of acid have reacted with one molecule of the protein. Hausmann finds for the amino-nitrogen of gelatin the value of 35·8 per cent. of the total nitrogen, which is far too high ; in more complete later determinations about 26 per cent. amino-nitrogen was found. Electrometric measurements show that the basic valency is reduced by 22 per cent. by removal of the amino-groups.

The NH-group of the peptide linkage is the first obvious direction in which to look for the origin of the combination of the desamino-proteins with acids. The acid can be added on at this point in just the same way as on to a terminal amino-group.



As will be discussed when treating of the combination of proteins with alkalis, the peptide linkage may change from the lactam (I.) into the lactim (II.) form, thus :—



It must, therefore, be emphasised that only the lactam form appears suitable for linkage with acids. This agrees with the determinations of M. Siegfried * according to which only the peptide linkage of polypeptides in which the lactam form is present can enter into combination with carbonic acid. Experience of the alkali proteins (see Chapter VIII.) indicates that the change from the lactam to the lactim form is a change requiring time ; consequently there is an increase in the combination of protein and alkali with time. The combination of albumin with acids, on the other hand, shows no notable alteration with time, at least in the concentrations usually employed. We can therefore conclude that this process occurs at lactam groupings which originally exist in the peptide linkages.

* R. H. A. Plimmer, "Chemical Constitution of the Proteins." Longmans. See Part II., p. 58, for the literature.

While the electrometric measurements show the existence of an ionised salt of desamino-glutin and acid, investigations of the viscosity indicate that no great increase in hydration results from the formation of these protein ions. The viscosity of desamino-glutin, in strong contrast with that of glutin, is not increased at all by combination with acid. We must therefore conclude that at least in the case of glutin the increase in hydration by formation of ions is bound up with the combination between acid and the terminal amino-groups. On the other hand, the cause of precipitation of desamino-glutin is clearly connected with its ionisation. Minimum precipitation coincides with the maximum of ionisation, as is shown by the following experiments on precipitation with phenol.

Table 37.

Precipitation of 0·75 per cent. Desamino-glutin + HCl by Phenol.

(10 *c.c. of the mixture treated with* 4 *c.c. of* 5 *per cent. phenol solution.*)

Concentration of HCl after addition of phenol.	Degree of precipitation.	Ionisation before addition of phenol ($C_H - C_{Cl}$).
·0036	± +	$3·99 \times 10^{-3}$
·0071	± ±	$6·0 \times 10^{-3}$
·0143	±	$6·22 \times 10^{-3}$
·0214	∓ Minimum	$5·07 \times 10^{-3}$
·0357	∓	$3·30 \times 10^{-3}$
·0714	± ±	$3·30 \times 10^{-3}$

Thus the heavy hydration of the albumin ions is in no wise a necessary preliminary to a decrease in precipitation of ionised protein salts.

According to the work of J. Christiansen* the hydrated ionised albumin is most attacked by pepsin, so that the first stages in the action of pepsin can be followed by noting the decrease in viscosity. The heavy hydration of the particles appears to be a favourable preliminary to hydrolytic decom-

* *Biochem. Zeitsch.*, 1912, **47**, 226.

position. The albumoses certainly give salts with acids which are much less hydrated, as can be seen by the much smaller rise in viscosity, although in this case also continued addition of acid gives rise to a maximum value for the viscosity.* It is only the ionic condition of the albumin and not the existing free H· ions which is important in the digestive action of pepsin on dissolved natural and uncoagulated albumin. In the same way the quantity of acid albumin formed by the action of pepsin passes through a maximum corresponding to the ionisation, when increasing quantities of acid are added. The following experiments by J. Christiansen show this effect.

Table 38.

1·3 per cent. Mutton Albumin + 0·01 per cent. Pepsin + HCl.

Total HCl.	Concentration of free HCl.	Degree of precipitation on neutralisation after one hour.
0	0	Clear
0·008	0	+
0·012	0	+ + + +
0·016	0	+ + + + +
0·018	Trace	+ + + + +
0·020	0·002 N	+ + + + +
0·022	0·004	+ + + + +
0·024	0·006	+ + +
0·028	0·010	+ + +
0·030	0·012	+ +
0·050	0·032	+ +
0·080	0·062	+

Finally, Christiansen has found that ionised albumin possesses the practically important property of being strongly adsorbed by filter paper, particularly in the case of the positive protein ions formed on combination with acids. This adsorption also passes through a maximum corresponding to the ionisation.

Some observations on the *acid-albumin* recently made by M. Adolf and E. Speigel † must be mentioned here. The denatured protein they employed, although insoluble in water, is

* Unpublished research of Pauli and P. Dukes. (See Table 40.)
† *Biochem. Zeitsch.*, 1920, **104**, 175.

soluble both in small quantities of acid and also in alkalis. It is prepared by treating albumin with hot dilute acid, but the alteration in chemical constitution in the albumin induced by this treatment is not at present known. The measurement of the capacity for combination with acid, however, gives a hint as to what has happened. As the following results show, this property is markedly greater than in the natural albumin :—

Table 39.

Acid-albumin (0·62 per cent.) + HCl.

Concentration of HCl.	0·008.	0·013.	0·018.	0·028.	0·048.	0·058 N.
Combined acid $n - \frac{C_H}{\alpha}$	$6{\cdot}17 \times 10^{-3}$	$8{\cdot}3 \times 10^{-3}$	$9{\cdot}73 \times 10^{-3}$	$10{\cdot}7 \times 10^{-3}$	$11{\cdot}4 \times 10^{-3}$	$12{\cdot}6 \times 10^{-3}$

According to Pauli and Hirschfeld, an albumin in 0·63 per cent. concentration takes up a maximum of $1{\cdot}0 \times 10^{-2}$ N HCl. per litre ; and although 1 gm. serum albumin combines with a maximum of $1{\cdot}66 \times 10^{-3}$ gm. equivalents of acid, the value for 1 gm. acid-albumin is $2{\cdot}06 \times 10^{-3}$ N HCl, or some 20 per cent. higher. We can conclude from this result that heating with dilute acid makes about one-fifth more of the basic valencies of the albumin operative, and that these remain permanently available for combination with acids. This type of alteration plays an essential part in the irreversible transformation which the acid-albumin has undergone. The capacity for combination with alkalis, on the other hand, remains unchanged by the transformation into acid-albumin. One gram of serum albumin combines with $2{\cdot}4 \times 10^{-3}$ gm. equivalents of alkali (see Chapter VII.), while measurements on acid-albumin give $2{\cdot}5 \times 10^{-3}$ N, or practically the same value.

If the degradation of the protein is carried beyond the stage of acid-albumin, greater changes in physico-chemical properties take place. Bugarsky and Liebermann (*loc. cit.*) made electrometric measurements on the capacity for combination with

acids of an albumose mixture made from Witte's peptone and freed as far as possible from salts and peptone by dialysis for five weeks. They found that the value calculated on unit dry weight was not less than that obtained for albumin. The collection of curves in Fig. 21, showing the viscosities of various protein derivatives in the same concentration, shows these variations in properties very clearly. The substances here included are a protalbumose made by the method of E. P. Pick from Witte's peptone, a mixture containing excess of glutoses made by heating glutin, freed from salts by dialysis, for an hour in an autoclave at 120°, and glutin heated for ten hours at 143° to break down the higher peptides. The viscosity curve of 1 per cent. serum albumin in the same range of acid concentrations is given for comparison.

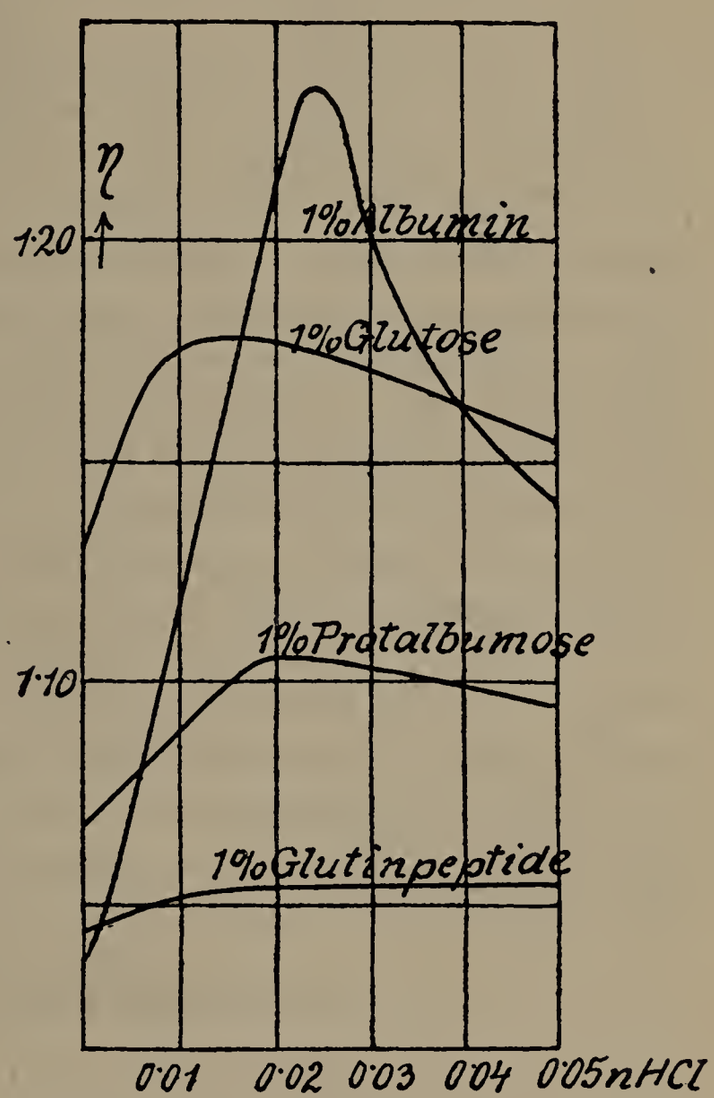


FIG. 21.—Combination of degraded proteins with hydrochloric acid.

The viscosity of protalbumose passes through a maximum on addition of acid, but the height of the curve is only one-third of that of the curve for albumin itself.

If one tried to plot the curve for 1 per cent. glutin on the same scale, this would not be possible even with ordinates four times as large. The great decrease in viscosity shown by the salt ions of glutose is thus very marked, for the maximum value scarcely reaches that of the albumin, while the glutin peptide gives a scarcely appreciable increase in viscosity on addition

Table 40.

Experiments on Viscosity at 25°.

Concentration of HCl.	Protalbumose 1%, η_1.	Glutin, 1 hour at 120°, η_2.	Glutin, 10 hours at 143°, η_3.
0·0 N	1·0673	1·1260	1·0465
0·01	1·0883	1·1700	1·0505
0·02	1·1017	1·1756	1·0507
0·05	1·0928	1·1516	1·0512
0·10	1·0825	1·1431	—

of hydrochloric acid. Simple amino-acids, such as glycocoll and alanin, give, with addition of acid, an increase in viscosity which can only just be recognised in 0·1 N solution. These results show that the linkage of amino-acids in albumin and the formation of albumin ions of high valency with acids has an extraordinarily important effect on the degree of hydration.

Hydration is known to decrease with rise in temperature, and this also appears to hold for the ions of the proteins. Pauli and O. Falek * found that the maximum viscosity of glutin chloride increased out of all proportion when the temperature was decreased. They found this by measurements on a 0·3 per cent. glutin which was treated with increasing quantities of hydrochloric acid within a temperature range of 30°—40°, whereby effects due to gelatinisation were excluded.

* *Biochem. Zeitsch.*, 1912, **47**, 276.

CHAPTER VII

SALTS OF ALBUMIN WITH BASES

THE albumins undergo fundamental chemical changes in the presence of high concentrations of acid at low temperatures; but with alkalis a much more marked sensitiveness is shown by most proteins even at room temperature. It is therefore from this point of view that work on the combination of alkalis with such proteins has to be carried out. As is well known, the more profound alteration of proteins by the action of strong acids and bases, when the so-called acid albumins and alkali albuminates are formed, is an irreversible change which takes time for completion. It can be recognised by the precipitation of these products when the solutions are neutralised. The change also proceeds further with a greater or less breaking down of the protein.

The electrometric method is the most important way of investigating quantitatively the combination of proteins with alkalis, in the same way as it has been found useful in the corresponding behaviour with acids. It was first applied to this problem, as to the latter, by Bugarsky and Liebermann. The procedure amounts to the determination of free hydrion, as from the dissociation equation of water at the temperature of experiment, $K_{H_2O} = C_H. \times C_{OH}$, the concentration of hydroxyl ions can be calculated, and from it the quantity of the alkali that has combined with the albumin.

In this way Bugarsky and Liebermann found that when increasing quantities of albumin were added to 100 c.c. of 0·05 N sodium hydroxide, complete combination with the alkali finally took place. This was also shown by the falling off of the depression of the freezing point of the solution, corresponding to a decrease in the total molecular concentration.

More recently Pauli and A. Spitzer * have carried out electro-

* Carried out in 1913–14, but not yet published.

Table 41.

Egg Albumin + 100 c.c. of 0·05 N NaOH.

(*g* = *gm albumin*, *p* = *percentage of alkali combined*, Δ = *depression of the freezing point.*)

g.	*p.*	Δ.
0	0	0·181
0·8	14·4	0·192
1·6	27·4	1·151
3·2	60·2	0·116
9·4	97·0	0·097

metric measurements over a wider range of concentrations of albumin and alkali, with the object of comparing the activity of various bases in combining with the protein. The calculation of the combined portion of the alkali is similar to that employed for acids; for strong bases the formula $n' = n - \frac{C_{OH}}{\alpha}$ is applicable, while for weak bases it can be derived by means of the dissociation constant from the expression

$$n' = \frac{K(n - C_{OH}) - C_{OH}^2}{K - C_H}.$$

These investigations involve the very probable assumption that the salts of albumin with weak bases are well ionised and give positive ions identical with those of the base itself. This conception is amply supported by the results of other experi-

Table 42.

Horse-serum Albumin 1 per cent.

Concentration of base (×N).	Combined fraction of the base per litre.				
	N NaOH.	N $Ba(OH)_2$.	N $(CH_3)_4NOH$.	N Piperidine.	N NH_4OH.
0·005	0·0049	0·0049	0·00498	0·0049	0·00498
0·01	0·00896	0·0093	0·00954	0·0097	0·00911
0·02	0·0159	0·0157	0·0164	0·0137	0·0122
0·03	0·0193	0·0190	0·0216	—	0·0139
0·05	0·0236	0·0221	0·0239	0·0171	0·0141

ments, such as those on electrical conductivity, electrophoresis, viscosity and precipitation. The results in Table 42, p. 94, were obtained with a constant concentration of albumin, the alkali content being varied.

It can be seen from these figures that, the quantity of albumin being constant, the combined portion increases with increasing concentration of alkali to a maximum, which then remains constant in excess of alkali. One gram of serum albumin combines with 0·0024 gm. equivalents of the base, a quantity notably greater than the value for combination with acids, which only amounts to 0·00166 gm. equivalents. Sodium hydroxide, baryta, and the very strong base tetramethylammonium hydroxide combine in almost equal quantities. Up to a concentration of 0·01 N both strong and weak bases combine to the same extent, that is, practically completely, with the 1 per cent. albumin; beyond this concentration combination falls off with decreasing strength of base. If isohydric concentrations of base are compared, combination with the weaker base is more complete, in analogy with the behaviour of the protein towards acids. Thus 0·05 N NH_4OH, with a hydroxyl content of $9{\cdot}3 \times 10^{-4}$, combines with practically the same quantity of albumin as a stronger base; but, in reality, 0·05 N ammonium hydroxide combines with 141×10^{-4}, or about five times as much albumin as the others, if the quantity is calculated on the number of free OH ions present.

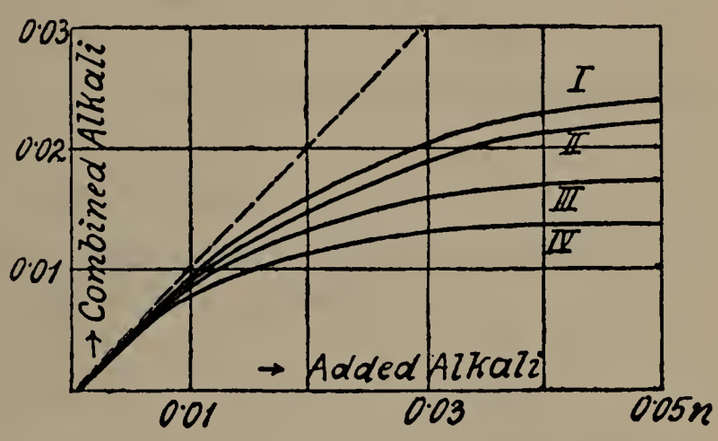


FIG. 22.

I. Tetramethylammonium hydroxide.
II. Barium hydroxide.
III. Piperidine.
IV. Ammonium hydroxide.

It is not possible to obtain a complete view of the behaviour of the salts of albumin with alkalis on ionisation owing to the lack of knowledge of the cation concentration. Thus indirect methods are very important in this case, as they give, when

combined, useful information on the dissociation of the protein salts.

Of these, the determinations of viscosity and of degree of precipitation by alcohol made by C. Schorr * on alkali-albumin will be considered first. The variation of these properties with time, which has been studied in other experiments, is allowed for by careful extrapolation of the original viscosity and by determining the quantity precipitated by alcohol immediately after the mixture was made up. In the following table the data obtained by Schorr are corrected in the light of recent electrometric measurements which allow for the viscosity due to the uncombined alkali. This slight alteration in the actual values does not, however, affect his general facts or the conclusions drawn from them.

Table 43.

Ox-serum Albumin free from Salts (1·15 per cent.) Relative Viscosity η at 25°.

NaOH.	η_{NaOH}.	η found.	η corrected.
0·0 N	1·000	1·072	1·072
0·005	1·001	1·086	1·086
0·04	1·010	1·115	1·109
0·05	1·012	1·270	1·264 *
0·1	1·024	1·265	1·247
0·5	1·109	1·320	1·217

These results, and those of similar experiments, show that that fraction of the viscosity of the alkali albumin which is due to the albumin passes through a maximum as the concentration of alkali is increased, and then, with further addition of alkali, falls off in value. With the more concentrated albumin solution quoted above, the maximum value is obtained in alkali of 0·05 N concentration.

Glutin also shows a maximum in viscosity, which occurs in still lower concentrations of alkali, and, owing to the high viscosity of the glutin solutions, the correction for viscosity of

* *Biochem. Zeitsch.*, 1912, **47**, 269.

the free alkali is superfluous. The following table gives the results of work by Pauli and O. Falek (*loc. cit.*) :—

Table 44.

Concentration of NaOH.	0·3% glutin at 35°.
0	1·1069
0·001 N	1·1943
0·002	1·2567
0·003	1·2798
0·004	1·2817
0·005	1·2870 *
0·01	1·2603

The effects are substantially the same as those found by Pauli and Handovsky with acid albumin, and can be referred to the maximum in the ionisation which later measurements by the electrometric and electrophoretic methods have certainly shown to exist. In applying the same conception to Schorr's results for viscosity, however, the objection may be urged that although they may have been carefully corrected for the variations in the albumin solution due to lapse of time, the decrease in viscosity in higher concentrations of alkali may be due to the formation of decomposition products of lower molecular weight. This objection, however, is not supported by the results of precipitation of alkali albumin by alcohol.

The fact that with increasing quantities of alkali the precipitation produced by alcohol passes through a minimum and then increases again cannot be explained except on the assumption of the formation of neutral particles. This result, moreover, is exactly analogous to that obtained with acid albumin, and as the precipitation was made immediately after mixing the alkali and the protein there is no time for any profound proteolysis. The minimum of precipitation practically coincides with the region of highest viscosity. The results given below were obtained by adding ten times the volume of alcohol to the mixture of alkali and protein, which is somewhat diluted by the water present in the 95 per cent. alcohol employed.

Table 45.

Ox-serum Albumin 1·15 per cent.

Concentration.	NaOH.	KOH.
0·001	± ± ±	±
0·002	± ±	±
0·003	±	±
0·005	± —	± —
0·01	± —	—
0·026	± — —	—
0·025	—	—
0·04	—	—
0·05	—	± —
0·1	±	±
0·2	+	+
0·3	± ± +	± ± +
0·5	± + +	± ± +

As distinct from the behaviour of proteins with different acids, the measurements of viscosity on combination of albumin with various bases agree with the electrometric measurements. The viscosity, and also the degree of combination, increases with increasing strength of the base employed. Fig. 23 shows the viscosity of albumin with various bases as investigated by Handovsky,* and a table of results showing the dissociation constant K_b and the relative times of flow in the viscometer for 0·30 N concentration of base is also inserted.

Table 46.

Ox-serum Albumin 1·4 per cent. at 25°.

Base (0·03 N).	$\frac{t}{t_0}$.	K_b.
Piperidine	1·238	$1·58 \times 10^{-3}$
Diethylamine . . .	1·228	$1·26 \times 10^{-3}$
Ethylamine . . .	1·179	$5·6 \times 10^{-4}$
Methylamine . . .	1·129	$5·0 \times 10^{-4}$
Trimethylamine . . .	1·145	$7·4 \times 10^{-5}$
Ammonia	1·123	$2·3 \times 10^{-4}$
Pilocarpine . . .	1·120	$1·0 \times 10^{-7}$
Pyridine	1·109	$2·28 \times 10^{-9}$

* *Biochem. Zeitsch.*, 1910, **25**, 510.

In spite of the lack of electrometric determinations of the cations, we must conclude from the results so far obtained that the ionisation of the alkali protein increases progressively with increase in the concentration of base until the highest value is reached, which corresponds to maximum viscosity and minimum precipitation by alcohol. The ionisation is repressed by excess of alkali, and there are thus the same differences in hydration and precipitation between the neutral and the ionised albumin as have already been noticed in the case of the acid proteins.

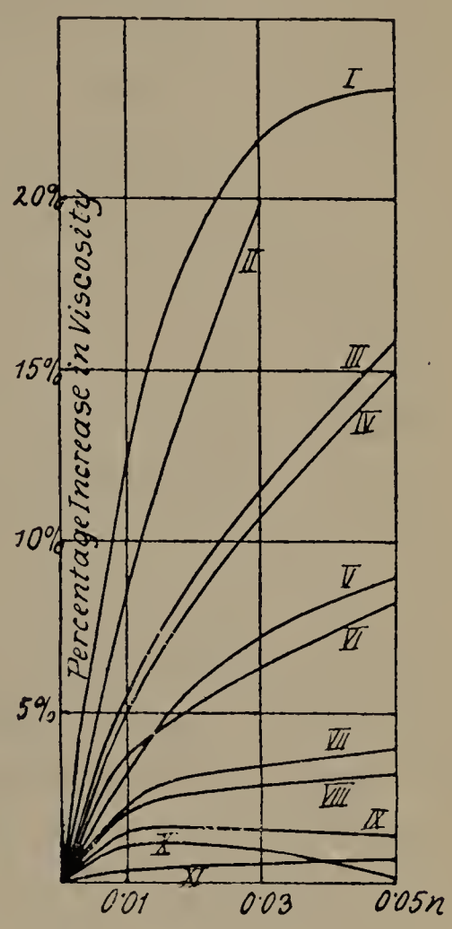


FIG. 23.

I. Tetraethylammonium hydroxide.
II. Sodium hydroxide.
III. Piperidine.
IV. Diethylamine.
V. Ethylamine.
VI. Methylamine.
VII. Trimethylamine.
VIII. Ammonium hydroxide.
IX. Nicotine.
X. Pyridine.
XI. Ecgonine.

In the electrophoresis apparatus, the protein portion of the alkali protein moves towards the anode, thus showing conclusively that negative protein ions are produced. In excess of alkali, electrometric measurements show no increase in combination, while there is much evidence for a suppression of the ionisation of the alkali protein. It is, therefore, extremely probable that the metallic ion of the alkali is the common ion in that and in the protein salt, which, according to this view, dissociates into a positive metallic ion and a negative protein ion. Further evidence for this conception, which is the opposite of that held by T. B. Robertson, is available, particularly from the results obtained for the electrical conductivity of the alkali proteins, which will be discussed later. As a consequence of this conception, the possibility of precipitation of the alkali albumin by excess of alkali has to be considered, and such a precipitation has actually been noticed by R. Wagner (*loc. cit.*, p. 78) in dialysed serum albumin in high concentra-

tions of alkali. It is analogous to the precipitation by excess of acid.

Table 47.

Ox-serum Albumin 1·03 per cent.
with various Alkali Concentrations.

NaOH.	18°.		KOH.	18°. After 5′.	36°.	
	After 5′.	After 24 h.			After 5′.	After 24 h.
1·18 N	—	—	1·33 N	—	—	—
2·35	—	—	2·66	—	—	—
3·53	—	—	3:99	—	—	—
4·70	—	—	5·32	+ +	+ +	±
5·88	—	—	6·65	± ± +	± ± +	± + +
7·06	+ +	±	7·98	± ± +	± ± +	± + +
8·23	+ +	±	9·31	± ± +	± ± +	± + ±
9·41	+ +	+ +	10·64	± ± +	± ± +	± + +

When strong bases of these concentrations are employed, complete formation of an alkali albuminate undoubtedly occurs eventually, but it is open to question whether the precipitate formed immediately is already a denatured product of some sort. The idea that it is the neutral portion of a protein salt which is precipitated by excess of alkali seems to meet this case as well. On standing for some time, decomposition of the precipitate sets in, it decreases in quantity, and finally dissolves completely.

The precipitation of albumin by alkali is notable in two ways. The product, which easily agglomerates to fibrous curds, differs from that of precipitation by acid in so far as it is easily soluble when water is added. In the second place, the precipitation by alkalis is but little affected by temperature changes over a considerable range, whereas the precipitation by concentrated acid is greatly favoured by rise in temperature, perhaps owing to an increase in the rate of the secondary and irreversible change in the albumin. In the case of weaker bases like piperidine and methylamine, the precipitate of serum albumin appears as an opalescent ring when a layer of the base is run on to serum albumin solution. Still weaker bases, such as triethylamine and trimethylamine, fail to give the reaction at all.

The study of the caseinates has been particularly useful in increasing our knowledge of the alkali proteins. Casein, the insoluble protein of milk, can be prepared in a state of great and constant purity practically free from ash by Hammersten's method. It gives an apparently well-defined salt when titrated with alkali and phenol phthalein, the hydrion concentration of the product approximating to absolute neutrality in a 1 per cent. solution. According to Laqueur and Sackur,* who made the first determination on the caseinates in which modern physico-chemical methods were employed, 1 gm. of casein is neutralised by 8·8 c.c. of N/10 sodium hydroxide, when phenol phthalein is used as indicator. In the light of more recent and more detailed researches † the physico-chemical properties of the caseinates deserve a short discussion at this point. In the first place, a fact which is extremely important and, indeed, decisive for the evaluation of the older observations, must be emphasised; that is, that even at great dilution solutions of neutral alkali caseinates show no hydrolytic dissociation which would disturb conductivity measurements. This is shown by electrometric tests made by Pauli and Matula, ‡ which gave no relative increase in the OH′ ion concentration on large and progressive dilution.

Table 48.

0·009 N NaOH (*saturated with casein*).

Dilution.	$P_{H^\cdot}$	$C_{H^\cdot}$
0	6·164	$6{\cdot}86 \times 10^{-7}$
1 : 1	6·212	$6{\cdot}14 \times 10^{-7}$
1 : 9	6·575	$2{\cdot}66 \times 10^{-7}$

These results give a firm foundation to the analogy between the behaviour of the alkali caseinates and that of the neutral salts of fairly strong acids. In consequence we can deal intelligently with the results obtained some time ago by Laqueur and Sackur, as also with the conductivity measure-

* *Beitr. z. Chem. Phys. Path.*, 1903, **3**, 193.
† Pauli and J. Matula, *Biochem. Zeitsch.*, 1919, **99**, 219.
‡ Pauli, *Biochem. Zeitsch.*, 1915, **70**, 489.

ments of Pauli, which agree well with the former. They can, in fact, be treated from the same point of view as obtains in the case of typical salts. These results, together with those obtained for the salts of albumin with acids, and those so far published dealing with the alkali proteins, all lead to the extremely probable conception that an ordinary metallic salt is formed, which dissociates into positive metal ions and negative polyvalent protein ions. Some very forcible facts supporting this view will be adduced. In the first place, owing to the absence of any marked hydrolysis, it is possible to extrapolate the value of λ_∞ from the values of the equivalent conductivity λ_V, obtained when caseinate solutions are progressively diluted. From the data of Laqueur and Sackur and the following tables, in which K is the specific conductivity, and V the volume in litres which contains the equivalent weight of sodium, the value of the equivalent conductivity λ_∞ for dilution $V = \infty$ could be extrapolated graphically. The values of the degree of dissociation α have been obtained in the usual way as the quotient of λ_V and λ_∞.

Table 49.

Sodium Caseinate at 25°.

Per cent. casein.	K.	V.	λ_V.	α.
2·84	$11·6 \times 10^{-4}$	40	46·5	0·575
1·42	$6·41 \times 10^{-4}$	80	51·3	0·637
0·71	$3·51 \times 10^{-4}$	160	56·2	0·70
0·355	$1·97 \times 10^{-4}$	320	63·0	0·787
0·1775	$1·09 \times 10^{-4}$	640	69·5	0·862

$\lambda_\infty = 81.$

Table 50 (Fig. 25, I.).

Ammonium Caseinate at 25°.

Per cent. casein.	K.	V.	λ_V.	α.
2·04	$10·7 \times 10^{-4}$	55·6	59·6	0·59
1·02	$5·9 \times 10^{-4}$	111·2	65·6	0·65
0·51	$3·21 \times 10^{-4}$	222·4	71·4	0·71
0·26	$1·73 \times 10^{-4}$	445·0	77·2	0·77
0·13	$0·96 \times 10^{-4}$	890·0	85·3	0·85

$\lambda_\infty = 104.$

For sodium caseinate the value $\lambda_\infty = u_{Na} + v_{Casein} = 81$ is obtained by the graphical method; for ammonium caseinate $\lambda_\infty = u_{NH_4} + v_{Casein} = 104$. Thus the difference, $u_{NH_4} - u_{Na} = 23$. The actual difference in the mobility of these two ions at 25° is 74·4 — 50·9 = 23·5. This agreement is seen to be a certain indication of the formation of typically ionised alkali caseinates. In the same way the almost equal values of the mobility of the potassium ion (u_K 18° = 64·6) and the ammonium ion (u_{NH_4} 18° = 64·0) agree well with the results obtained for the equivalent conductivity of ammonium and potassium caseinates.

Table 51.

Casein + KOH (*Pauli*).			Casein + NH_4OH (*Laqueur and Sackur*).		
Per cent. casein.	K × 10^5.	λ_v.	Per cent. casein.	K′ × 10^5.	$\lambda_{v'}$.
1·0	66·96	66·96	1·02	59·0	65·6
0·5	35·38	71·66	0·51	32·1	71·4
0·25	19·49	77·96	0·26	17·3	77·2

As the difference of the equivalent conductivity,* $\lambda = \alpha(u + v)$, of two caseinates with different ions is, *e.g.*, $\lambda_{K.Cas} - \lambda_{Na.Cas} = \alpha(u_K - u_{Na})$, the value calculated for 1 per cent. caseinate solutions, the degree of dissociation being 0·66, is $\lambda_{K.Cas} - \lambda_{Na.Cas} = 13·9$. Direct experiment gives the value 14·13 (Pauli, *loc. cit.*, p. 499). These results are undeniable evidence of the existence of alkali caseinates, which dissociate into alkali metal ions and caseinate ions.

The mobility of the caseinate ion, calculated from the λ_∞ of the alkali caseinate, is $v_{Cas} = 30·1$. This value is entirely within the range of mobility shown by numerous organic ions. We shall return later to the importance of high valency and large atomicity in regard to the mobility of the ion.

With a knowledge of the equivalent conductivity of the caseinates and of their regular behaviour, it is possible to

* The pertinent observation of Pauli (*loc. cit.*) that a practically complete dissociation of the caseinate occurs is justified by a consideration of the degree of dissociation.

evaluate the valency of the caseinate ion. Taking the result of Laqueur and Sackur that 1 gm. of casein is neutralised by 0·881 millimols of sodium hydroxide, the equivalent weight of casein is $\frac{1,000}{0·881} = 1,135$. This value, in the light of more recent work (see below), appears to be somewhat too high. The molecular weight of casein is certainly greater than this, but will be an integral multiple of this number.

From consideration of a large number of mono- and polybasic organic acids (up to a basicity of five), W. Ostwald has deduced the rule that the increase in conductivity when equivalent weights of the sodium salts are diluted is proportional to the valency of the anions. The relation is particularly simple if the increase in conductivity at 25° for volumes of 32 litres and 1,024 litres is considered. The difference $\lambda_{1024} - \lambda_{32} = n \times 10$, where n is the valency of the anion. The following table by W. Ostwald shows over what a wide range the rule holds good.

Table 52.

Sodium salts of	$\lambda_{1024} - \lambda_{32}$.	
Quinolinic acid	19·8	= 2 × 9·9
Phenyl pyridine carboxylic acid	18·1	= 2 × 9·1
Pyridine tricarboxylic acid [1, 2, 3]	31·0	= 3 × 10·3
Pyridine tricarboxylic acid [1, 2, 4]	29·4	= 3 × 9·8
Methyl pyridine tricarboxylic acid	30·8	= 3 × 10·3
Pseudo-aconitic acid	29·6	= 3 × 9·9
Pyridine tetracarboxylic acid	40·4	= 4 × 10·1
Propargylene tetracarboxylic acid	41·8	= 4 × 10·45
Pyridine pentacarboxylic acid	50·1	= 5 × 10·0

For sodium caseinate, the values $\lambda_{32} = 45$, and $\lambda_{1024} = 75$, are obtained graphically when v and λ_V are used as co-ordinates in place of $1/V$ and λ_V. The difference is 30 or 3 × 10, and thus in this salt the caseinate is by Ostwald's rule tribasic. The molecular weight itself therefore becomes 3,400. Laqueur and Sackur believed the casein ions to have a low mobility, and the use of the relative decrease in conductivity, $(\lambda_1 - \lambda_2)/\lambda_2$, to be essential when calculating the basicity, and therefore estimated the basicity of the casein to be 4—6. The normal behaviour

of casein with regard to the equivalent conductivity, however, leaves no doubt that this is an ordinary case for the application of Ostwald's rule. Further, the relation emphasised by Bodländer and Storbeck that the degree of dissociation of a salt of $(n + 1)$ ions is the nth power of the degree of dissociation of a salt of $(1 + 1)$ ions, although of restricted application, gives the value $n = 3$ for the valency of the caseinate ions when sodium caseinate is compared with sodium bromide and sodium chloride.

The experimental determination of the valency of the caseinate ion by the osmotic pressure presents no mathematical difficulty when the degree of dissociation is known. It is, however, rendered impossible by disturbances due to hydrolysis which, in consequence of the constant dialysis of even small quantities of alkali, increases so much that usually after twelve hours a separation and even precipitation of casein set free by hydrolysis makes its appearance. Under these conditions, the molecular weight calculated from the osmotic pressure is invariably too high.

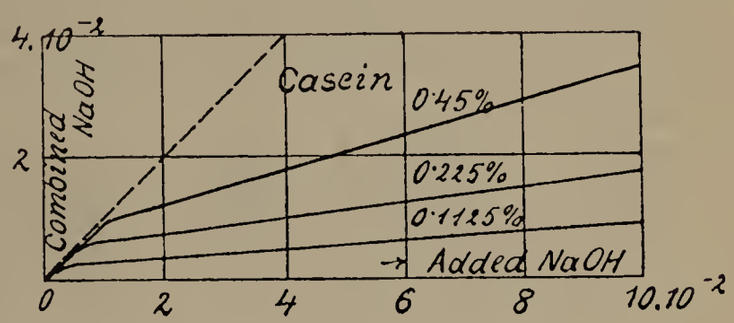


FIG. 24 (Table 53).

Electrometric measurements have shown that of the proteins thus investigated casein is distinguished by a quite extraordinary capacity for combining with alkali in excess of the alkaline solution. A value of 10 milligram equivalents of alkali hydroxide per gram of casein can be attained, corresponding to a value of 100 for the equivalent of the casein; and so, as a valency of three is deduced from the experiments in which a gram of casein combines with 0·9 millimols of sodium hydroxide, the maximum valency displayed when saturated with alkali is, in round figures, thirty.

This high value for the combining capacity of casein is obtained from the work of Pauli and F. Kryz quoted below, in which, however, there is some uncertainty in the values for

concentrations of more than 0·06 N alkali. It can be seen from Fig. 24 that a range of practically complete combination follows on increasing alkali content, as new acid valencies of the casein come into play almost in proportion to the quantity of alkali present.

Table 53 (Fig. 24).

Stock Solution : 4·5 gm. Dry Casein in 500 c.c. 0·01 N NaOH.

(*Electrometric determination made one hour after mixing.*)

0·45 per cent. casein.		0·225 per cent. casein.		0·1125 per cent. casein.	
N NaOH.		N NaOH.		N NaOH.	
Added.	Combined.	Added.	Combined.	Added.	Combined.
0·025	0·0124	—	—	—	—
0·045	0·0189	0·0425	0·0085	0·04125	0·00425
0·065	0·0257	0·0625	0·011	0·06125	0·0047
0·085	0·0307	0·0825	0·139	0·08125	0·0051
0·105	0·0376	0·1025	0·167	0·10125	0·0064
0·125	0·041	—	—	0·12125	0·00778

The original neutral alkali caseinate made by Laqueur and Sackur by titration with phenol phthalein contained 0·9 (or accurately 0·881) millimols of alkali per gram of dry casein ; whereas the work of Pauli and J. Matula gives the result that when excess of casein is shaken at 20° in 0·01 N sodium hydroxide about 2 gm. of casein dissolve in each 100 c.c. The fairly clear and stable solution obtained in this way contains only 0·5 millimols of alkali per gram of casein.

This somewhat complex behaviour becomes much clearer if we start from this caseinate saturated with casein. In the first place it is possible to dilute the above solution with half its volume of alkali of the same concentration without any considerable displacement of the H ion concentration from the neutral point. A series of such results is given in Table 54 on p. 107.

It follows from these experiments that all caseinates containing between 1 and 2 gm. of casein per millimol of alkali behave

Table 54.

(*Measurements at* 20° ; *content of alkali* 0·01 N *in all cases.*)

Per cent. casein.	P_H.	C_H or C_{OH}.
1·92	6·308	$4·92 \times 10^{-7}$ N H·
0·82	7·964	$7·92 \times 10^{-7}$ N OH′
2·01	6·387	$4·00 \times 10^{-7}$ N H·
1·33	7·143	$1·20 \times 10^{-7}$ N OH′
1·01	7·771	$5·10 \times 10^{-7}$ N OH′
0·905	7·294	$1·70 \times 10^{-7}$ N OH′

as neutral salts, and thus the caseinate obtained by Laqueur and Sackur on titrating casein in presence of phenol phthalein represents only one arbitrarily selected member of this continuous series. The particular case they investigated certainly lies near that end of the series where the concentration of

Table 55 (Fig. 25, Curve II.).

($t = 20°$, $C_H = 4·92 \times 10^{-7}$.)

% casein.	κ.	V.	λ.
5·76	—	33·3	40·7 *
1·92	$50·6 \times 10^{-5}$	100	50·6
0·96	$27·7 \times 10^{-5}$	200	55·4
0·48	$14·9 \times 10^{-5}$	400	59·6
0·24	$8·4 \times 10^{-5}$	800	67·2
0·12	$4·6 \times 10^{-5}$	1600	73·6
—	—	∞	79·0

Table 56 (Fig. 25, Curve III.).

($t = 25°$.)

% casein.	κ.	V.	λ.
2·88	—	33·3	44·1 *
0·96	$52·7 \times 10^{-5}$	100	52·7
0·48	$28·7 \times 10^{-5}$	200	57·4
0·24	$15·7 \times 10^{-5}$	400	64·8
0·12	$9·0 \times 10^{-5}$	800	72·0
0·06	$4·9 \times 10^{-5}$	1600	78·4
—	—	∞	83—84

protein is least. An increase in alkali content at this point leads to increasing quantities of it remaining in the free state.

The results shown in Tables 55 and 56 give the conductivity data for increasing dilution of the casein-saturated caseinate with water. The original solution was made by shaking with 0·03 N sodium hydroxide. Similar figures are given for such a solution diluted with an equal volume of the alkali, forming a caseinate half saturated with casein.

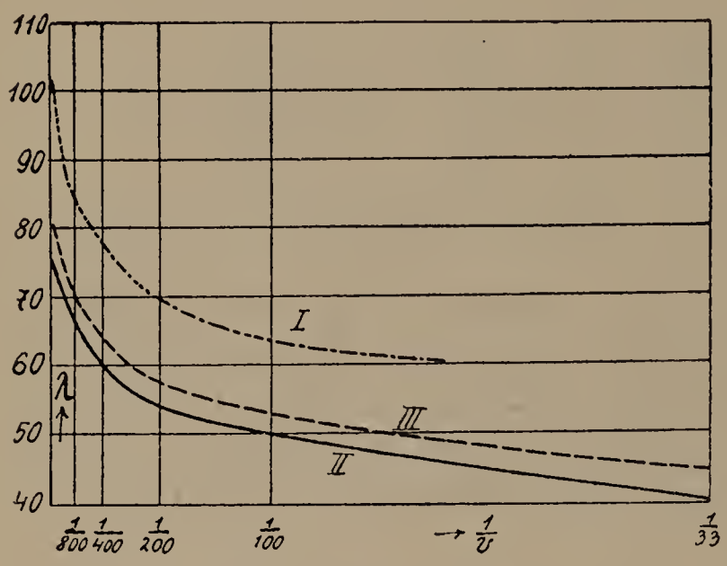


Fig. 25.

The conductivity curves (Fig. 25) run parallel to each other. The λ_∞ of the salt saturated with protein has the value 79, corresponding to $V_{\text{Caseinate}} = 28$; the λ_∞ of the half saturated caseinate has the value 83—84, which leads to a value for mobility of the caseinate ion of 32·5. Thus the mobility is 14 per cent. less in the saturated solution than it is in the latter case.

The application of Ostwald's rule gives $\lambda_{1024} - \lambda_{32} = 3 \times 10{\cdot}1$, or a trivalent casein ion, in both cases. Hence when one passes from the half saturation to the almost complete saturation of the alkali salt with casein there is no change in valency, and only a moderate decrease in mobility of the ions.

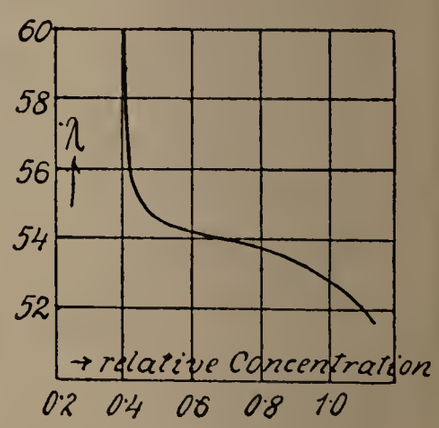


Fig. 26.

Of course an increase in valency would cause a great rise in the ionic conductivity (see p. 125). We should expect such an increase in valency from the curves showing the course of combination of alkali and casein, when the continued addition of alkali causes new acid valencies of the casein to become active.

If to a solution of alkali caseinate saturated with casein

increasing quantities of the same solution of base are added, there is a moderate increase in conductivity up to a dilution of one half. Beyond that point the equivalent conductivity increases rapidly owing to increasing valency and consequent increase in mobility of the ions. This is shown by the following results, in which the free alkali is taken into consideration. The corresponding curve (Fig. 26), which must be read from right to left, shows a sharp break when the dilution becomes greater than one half (between 0·4 and 0·6).

Table 57 (Fig. 26).

2·01 *per cent. dry casein in* 100 *c.c.* 0·01 N *NaOH, progressively diluted with* 0·01 N *NaOH.*

% casein.	Relative concentration.	λ.	P_H.	C_H or C_{OH}.
2·01	1·00	52·6	6·396	$4·10 \times 10^{-7}$ N H·
1·33	0·66	54·1	6·91	$1·2 \times 10^{-7}$ N OH′
1·00	0·5	54·6	7·771	$5·1 \times 10^{-7}$ N OH′
0·90	0·45	56·4 [55·94] *	8·3	$1·7 \times 10^{-6}$ N OH′
0·80	0·4	60·3 [59·76] *	9·698	$2·0 \times 10^{-5}$ N OH′
0·50	0·25	78·6	—	—

* Corrected values.

These results all become explicable without difficulty when the following view is adopted. A neutral salt exists, composed of approximately 1 millimol of alkali and 1 gm. of casein, the ion being trivalent, viz. :

Na_3 . (Caseinate)‴. . . . . (I.)

This salt can form a typical complex salt by addition of a molecule of casein :

Na_3 . (Caseinate)‴ . (Casein) . . . . . (II.)

the valency remaining unaltered. Addition of alkali converts salt (II.) into salt (I.) with moderate increase in conductivity, the valency remaining constant. The equivalent weight of casein calculated from these results is 1,000, somewhat lower than the value obtained by Laqueur and Sackur, giving a molecular weight of 3,000. Probably the alkali salts of the globulins and of the acid-albumins behave in a similar way to that of casein, which, however, is particularly suitable for experiment owing to the abundance of its acid valencies.

CHAPTER VIII

ALTERATIONS IN STATE OF THE ALKALI PROTEINS WITH LAPSE OF TIME

MORE detailed experiments have soon shown that the physico-chemical properties of alkali protein solutions alter with lapse of time in a remarkable manner. This occurs even at room temperature and on addition of quite low concentrations of alkali when no question of breaking down of the protein can arise. The compounds of acids and proteins, on the other hand, are remarkably stable under the same conditions. These changes of state with time were first investigated in a detailed manner by C. Schorr * at this Institute. He found that the viscosity of an approximately 1 per cent. serum albumin in alkali concentrations of 0·04 N upwards rose with time to a maximum value and then decreased again. The following table gives typical results for the viscosity at 24·5°.

Table 58.

Time.	N NaOH.		Time.	N NaOH.		Time.	N NaOH.	
	0·083.	0·25.		0·083.	0·25.		0·083.	0·25.
Original value (extrapolated)	1·351	1·378	– 28′ 25″	1·456	—	2 hr. 37′ –	1·595	—
5′ 35″	1·374	—	– 30′ –	—	—	2 hr. 51′ 30″	1·612	—
7′ 30″	—	1·451	– 31′ 40″	1·471	—	3 hr. 5′ –	1·594	—
8′ 20″	1·386	—	– 33′ 30″	—	1·594	3 hr. 41′ –	1·586	—
11′ 10″	1·397	—	– 36′ 30″	—	1·624	4 hr. 13′ –	1·580	1·465
13′ 30″	—	1·508	– 37′ 40″	1·488	—	4 hr. 45′ –	—	1·451
14′ –	1·411	—	– 40′ 30″	—	1·607	5 hr. 20′ –	—	1·425
14′ 30″	—	1·517	1 hr. 9′ 30″	1·542	1·581	5 hr. 55′ –	—	1·412
16′ 45″	1·418	1·539	1 hr. 27′ 30″	1·567	—	24 hr. – –	1·512	—
19′ 40″	1·430	1·554	1 hr. 30′ –	—	1·567	33 hr. – –	—	1·318
21′ 30″	—	1·563	1 hr. 48′ 30″	—	1·557	46 hr. – –	—	—
22′ 35″	1·441	—	1 hr. 54 –	1·578	—	—	—	—
27′ 30″	—	1·583	2 hr. 22′ –	1·588	—	—	—	—

* *Biochem. Zeitsch.*, 1911, **37**, 424.

A lower temperature delays the alteration in viscosity considerably and increases the maximum value, probably owing to inhibition of the processes which find their expression in the decrease of viscosity.

The variations in viscosity correspond to a change in conductivity with time ; this property suffers a continuous decrease, and no point when the decrease is reversed corresponding to the alteration in viscosity can be recognised, as the following table shows.

Table 59.

Specific Conductivity of Alkali Albumin ($K \times 10^6$).

Time after mixing (extrapolated).	0·0125 N NaOH.	0·0463 N NaOH.	0·125 N NaOH.
– 0′ . . .	1,465	7,188	$2,325_8$
– 12′ . . .	—	$7,165_6$	—
– 13′ . . .	—	—	$2,319_6$
– 14′ . . .	—	$7,161_4$	—
– 15′ . . .	$1,458_6$	—	$2,317_2$
– 17′ . . .	$1,455_1$	—	$2,316_8$
– 18′ . . .	—	$7,152_6$	—
– 19′ . . .	—	—	—
– 20′ . . .	—	—	$2,314_4$
– 23′ . . .	$1,454_3$	—	$2,312_9$
– 25′ . . .	—	—	$2,312_5$
– 27′ . . .	—	$7,132_6$	$2,311_5$
– 30′ . . .	—	$7,129_7$	—
– 31′ . . .	—	—	—
– 36′ . . .	—	—	—
– 39′ . . .	—	$7,118_2$	$2,306_8$
– 42′ . . .	—	—	$2,305_9$
– 44′ . . .	—	$7,098_3$	—
– 45′ . . .	$1,448_1$	—	—
– 56′ . . .	—	—	—
– 60′ . . .	—	—	—
1 hr. 10′ . . .	$1,442_4$	—	—
1 hr. 19′ . . .	—	—	—
1 hr. 24′ . . .	—	—	—
2 hr. 4′ . . .	—	—	—
3 hr. 6′ . . .	$1,434_6$	—	—
6 hr. 25′ . . .	—	$6,932_7$	$2,273_8$
7 hr. 35′ . . .	—	$6,758_8$	—
16 hr. 35′ . . .	—	—	—
16 hr. 48′ . . .	—	—	—
20 hr. – . . .	$1,338_4$	—	—
24 hr. – . . .	—	$6,380_6$	2,241
29 hr. – . . .	—	$6,290_3$	—
51 hr. – . . .	$1,250_2$	—	—
55 hr. – . . .	—	—	—

The 1·18 per cent. serum albumin used by Schorr shows a minimum in the quantity precipitated by alcohol (Table 45), together with a maximum of viscosity and of conductivity in an alkali concentration of approximately 0·05 N. The following tables show this property.

Table 60.

η_1 = viscosity of the NaOH ; η = viscosity of ox-serum albumin + NaOH ; $\eta_{calc.}$ = calculated viscosity of NaOH + albumin.

NaOH.	η_1	η.	$\eta_{calc.}$	$\eta - \eta_{calc.}$
0·0 N	0·000	1·072	1·072	0·000
0·005	1·001	1·086	1·073	0·013
0·04	1·01	1·115	1·082	0·033
0·05	1·012	1·270	1·084	0·186
0·1	1·024	1·265	1·096	0·169
0·5	1·109	1·320	1·181	0·139

Table 61.

Specific Conductivity of Alkali Albumin at 25° ($\times 10^6$).

K_1, of the NaOH ; K, of the ox-serum albumin + NaOH (observed) ; $K_{calc.}$, of the albumin + NaOH (calculated).

NaOH.	K_1.	K.	$K_{calc.}$	$K_{calc.} - K$.
0·0 N	0	14_2	14_2	0
0·005	111_9	41_7	126_1	84
0·0125	273_9	146_5	288_1	142
0·037	783_7	542_5	797_9	255
0·0463	965_5	718_8	979_1	261
0·1	$2{,}016_5$	1,833	2,031	198
0·125	2,491	2,326	2,505	179
0·25	4,872	4,806	4,886	80

This maximum of albumin ions corresponds with the fact that the increase in viscosity with time also reaches a maximum in this concentration of alkali. The same holds good of the decrease in conductivity of alkali albumin mixtures with lapse of time. Compare, for example, the following table with the preceding one.

Table 62.

Diminution of Conductivity with Time as related to concentration of Alkali (1·1 per cent. Ox-serum Albumin).

NaOH.	Extrapolated original value $K_0 \times 10^6$.	Conductivity after 7 hr., $K \times 10^6$.	Percentage diminution $\frac{K_0 - K}{K} \times 100$.
0·005 N	417	416	0·0 %
0·0125	1,465	1,412	3·6
0·037	5,425	5,129	5·6
0·0463	7,188	6,763	5·9
0·1	18,330	17,630	3·8
0·125	23,260	22,690	2·5
0·25	48,060	47,650	0·9

The variation in viscosity and conductivity of a 1 per cent. ox-serum plus 0·125 N sodium hydroxide is most marked in the first six hours. Schorr was unable to discover during this time any difference in molecular concentration by determining the depression of the freezing point. This method, however, is inadequate to detect the small changes which might occur.

The changes in viscosity with time lend considerable probability to the conclusion that the hydration of the albumin ions and perhaps also of the neutral particles of alkali protein first increases with time, and then decreases again. The fact that a relative maximum occurs, with increasing alkali content, at a point corresponding to the maximum ionisation of the alkali protein, shows that the ions are principally concerned in this effect. The rise in hydration of the particles of albumin accounts for the decrease in electrical conductivity as the mobility of the protein ions is thereby diminished. Apart from the fact that a parallel between viscosity and conductivity is missing over a considerable range, other evidence is all in favour of the possibility of an increasing combination of the protein with the alkali as time goes on. This process leads to the disappearance of the particularly mobile hydroxyl ion, and hence to a continuous decrease in conductivity. It is only necessary now to demonstrate that the quantity of alkali which enters into combination actually increases as time proceeds, and this is shown by the following experiments of Pauli and Spitzer (*loc. cit.*).

Table 63.

Variation in combination of Alkali and Albumin with Lapse of Time.

After time.	P_H.	P_{OH}.	C_{OH} combined per litre.
0·02*N NaOH*; 1·26 % *serum albumin.*			
15′	12·003	2·137	$1·203 \times 10^{-2}$
80′	11·987	2·153	$1·232 \times 10^{-2}$
280′	11·941	2·199	$1·309 \times 10^{-2}$
425′	11·899	2·241	$1·373 \times 10^{-2}$
25 hr.	11·834	2·306	$1·460 \times 10^{-2}$
0·05*N NaOH*; 1·26 % *serum albumin.*			
15′	12·600	1·540	$1·672 \times 10^{-2}$
160′	12·553	1·587	$2·018 \times 10^{-2}$
400′	12·499	1·641	$2·366 \times 10^{-2}$
24 hr.	12·435	1·705	$2·726 \times 10^{-2}$

The alkali caseinates also show changes in physico-chemical properties with lapse of time, the viscosity variations being particularly interesting. Laqueur and Sackur made the far-reaching generalisation, which later work has shown to be unexceptionable, that the ions of the protein are responsible for high viscosity. It is based on two observations: first, the increase in viscosity of caseinates on addition of alkali, and secondly, the decrease when sodium chloride is added to sodium caseinate. These authors explained the latter effect by the action of the common ion of the sodium chloride in suppressing the ionisation of the sodium caseinate. This loses its cogency since it has been shown by Pauli and Handovsky (*loc. cit.*) that any salt, whether it contains a common ion or not, produces this result with alkali proteins. Further, investigation has also shown the viscosity of the alkali caseinates to be less simple than this, and in particular to be complicated by changes with lapse of time. The experimental results * in Table 64 show this behaviour clearly.

The viscosity of the caseinate actually passes through a maximum value as the concentration of alkali is increased, but the high viscosity falls off with progress of time, and in five to ten

* *Biochem. Zeitsch.*, 1915, **70**, 489.

Table 64.

Sodium Hydroxide + 0·5 per cent. Casein at 25°.

Concentrations of NaOH (× N).	0·0045.	0·0055.	0·0095.	0·0145.	0·0185.	0·0195.
Immediately	1·1185	1·1849	1·2028	1·2064	1·1939	1·1885
After 5′ .	—	1·1723	1·2028	1·2064	1·1939	1·1885
,, 10′ .	1·1023	1·1454	1·1975	1·1993	1·1939	1·1885
,, 15′ .	—	—	1·1849	1·1939	1·1939	1·1885
,, 20′ .	—	1·1383	1·1777	1·1849	1·1939	1·1885
,, 30′ .	1·0933	1·1310	1·1759	1·1849	—	—
,, 40′ .	—	1·1221	1·1723	1·1795	—	—
,, 50′ .	—	1·1149	1·1634	1·1705	—	—
,, 60′ .	—	1·1149	1·1580	1·1508	—	—
,, 70′ .	—	—	1·1490	1·1490	—	—
		After 17 *hr.* 1·1041	*After* 5 *hr.* 1·1041	*After* 8 *hr.* 1·1039	*After* 45 *hr.* 1·1039	*After* 30 *hr.* 1·0956

hours reaches a fairly constant value which is independent of the alkali concentration. An increase in viscosity preceding the decrease, such as appears when time observations are made on alkali albumins, is not noticeable in the case of casein. On the other hand the rapid decrease in electrical conductivity is also displayed by casein ; for example, with 0·5 per cent. caseinate plus 0·025 N sodium hydroxide, it falls to half the original value after forty-eight hours (see the same paper, Table 4).

The increase with time of the quantity of alkali which enters into combination is also very clear with alkali caseinates, as the following table shows.

Table 65.

Temperature 18°—21°. 0·5 *per cent. casein in all cases.*

Time in days.	0·02 N NaOH combined.	0·04 N NaOH combined.	0·08 N NaOH combined.
0	0·0124 N	0·0177 N	0·0307 N
2	0·0134	0·0186	0·0315
3	0·0134	0·0202	0·0335
5	0·0142	0·0202	—
6	0·0143	—	—
7	0·0159	0·0215	—
8	0·0152	0·0221	—

An alteration of the freezing point depression with time can be noted in such solutions when the alkali content is high. This result corresponds to a decrease in total molecular concentration, and, like the fall in conductivity with time, probably finds its explanation in the increased combination of alkali with casein, as proved electrometrically. In any case, the observation of a decrease in molecular concentration of the alkali caseinates is evidence against the existence of any considerable hydrolytic decomposition of the protein.

These observed results can be considered from the chemical point of view by dealing with the question of the structure of the salts of proteins with bases. The earlier workers, particularly Liebermann and Bugarsky, held the view that proteins form typical metallic salts with bases. This conception was adhered to by W. B. Hardy and by Pauli, and expressed in the following equation, in which the free carboxyl groups react as acid valencies with formation of a negative protein ion :

$$R\begin{cases} NH_2 \\ COOH \end{cases} + NaOH \longrightarrow R\begin{cases} NH_2 \\ COO\,.\,Na \end{cases} + H_2O.$$

T. B. Robertson, also, is entitled to the credit of emphasising the importance of the peptide linkage as the source of basic and acid valencies, and bases his theory on the following scheme, in which the peptide group reacts in its enol form :

$$\ldots\ldots{-}COH:N{-}\ldots\ldots + NaOH \longrightarrow \ldots{-}CONa^{++} + N''{-}\ldots$$

$$\begin{matrix} OH & H \end{matrix}$$

According to this scheme, the protein is ionised by rupture at the peptide linkage with formation of two oppositely charged protein fragments. Further, according to Robertson, diamino- and dicarboxyl-groups play a leading part, so that, doubling the previous equation, the formation of two quadrivalent radicles from a double peptide linkage is seen to occur :

$$R\begin{cases} COH:N{-} \\ COH:N{-} \end{cases} + 2KOH \longrightarrow R\begin{cases} COK^{++} \\ COK^{++} \end{cases} + \begin{matrix} H\diagdown\diagup OH \\ N''{-} \\ N''{-} \\ H\diagup\diagdown OH \end{matrix} \ldots\ldots$$

Protein salts of the Robertson type, therefore, give no free metal ions, but only equal numbers of oppositely charged protein ions. When investigated in the electrophoresis apparatus, however, the protein salts with alkali show only negative protein ions, in the same way as when acid is added, migration to the cathode alone occurs. In this place, without going into the arguments which Robertson uses to support his theory, we will give a summary of the facts which tend to show that alkalis form regular salts with proteins, the latter behaving as polybasic acids.

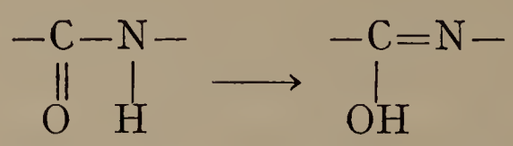
1. The protein part of the alkali protein migrates to the positive pole, while the alkali content increases at the negative pole.
2. The viscosity of solutions of the protein salt passes through a maximum value corresponding to a repression of the ionisation of the alkali protein in excess of alkali —a behaviour exactly parallel to that of acid albumin in excess of acid.
3. The difference in electrical conductivity between sodium caseinate and ammonium or potassium caseinate corresponds to the difference in mobility of the sodium ion compared with the ammonium or potassium ion. This behaviour is compatible only with the existence of free metal ions.
4. When casein is added to sodium hydroxide solution a fall in the total molecular concentration of the latter occurs which can be demonstrated by the diminution of the freezing point depression. This effect is produced by the polyacid character of the casein, as a result of which only one polyvalent casein ion appears when several OH ions of the alkali are replaced.

In the proteins that have been studied the basic constant K_b is large compared with the acid constant K_a. In dialysis of natural albumin the alkali is the more difficult to remove, so much so that at one stage in the dialysis the protein reacts as an alkali protein.

The carboxyl-groups at the end of the chains in the protein molecule primarily provide the acid valencies for combination

with alkali. These are the first to be protected by methylation of the protein, but even then a marked capacity for combination with alkali persists. For, besides the terminal carboxyl groups, the peptide linkages can also react as acid valencies. There is no doubt that by transformation into the lactim form (II.), thus :—

I. Lactam or keto form



Lactim or enol form II.

it can function as an acid group with a hydrogen atom replaceable by metals. It is quite unknown how far the peptide linkages in the various proteins are originally in form I. or form II., but there is a certain amount of evidence that the enol form of some part of the peptide complex arises only as a secondary effect of the addition of alkali. This view is supported by the changes of hydration and conductivity with time, which, *ceteris paribus,* distinguish the alkali proteins from the acid proteins and which appear to be essentially a consequence of increased combination with alkali, which in turn is accomplished by rearrangement into the lactim form. In this way albumin can be regarded as a pseudo-acid as defined by Hantzsch.

The occurrence of isomerism by transformation of the peptide linkage has been recognised for some time in the case of the di- and tripeptides. H. Leuchs and W. Manasse * showed, in 1907, that the ester of carbethoxyglycylglycine on hydrolysis with alkalis passes from the lactam form I. into the lactim form II. Here by the action of alkalis, therefore, the resulting glycylglycincarboxylic acid on esterification gives an ester isomeric with the original substance.

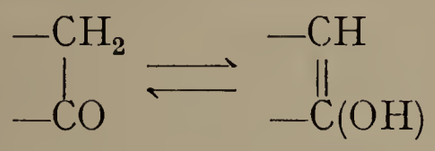
Leuchs and La Forge † a year later found the same effect with diglycylglycin ester, in which both peptide linkages undergo the change.

In the supposed transformation of the peptide linkage in higher polypeptides with which we are concerned, no asymmetrical carbon atom is directly engaged, and we were in fact

* *Ber.*, 1907, **40**, 3235.
† *Ber.*, 1908, **41**, 2586.

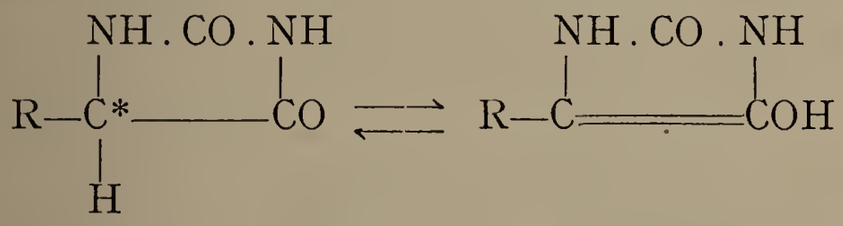
unable to observe any alteration of the optical rotation with lapse of time in glutin solutions in concentrations of alkali up to 0·1 N.

On the other hand, a very marked falling off of optical rotation with time has been observed as a result of the alterations of state which take place in clupein and in glutin (A. Kossel *). An explanation has been furnished by H. D. Dakin.† It is here a question of the change from the keto to the enol form by the action of strong alkali on the group :



I. . . . . II.

In this case an asymmetric carbon atom is concerned, so that, as the following example (hydantoin) shows, an optically inactive form II. results :



I. II.

Dakin's representation of the rearrangement shows the carbon atom next the peptide linkage behaving as an asymmetric carbon atom, but the formation of the hydroxyl group with acid properties follows as in the lactim form in amino-acid chains. For clearer comparison we give below the scheme of Leuchs of the transformation at the peptide linkage and that of Dakin of the change at neighbouring linkages in the case of a tripeptide.

Leuchs.

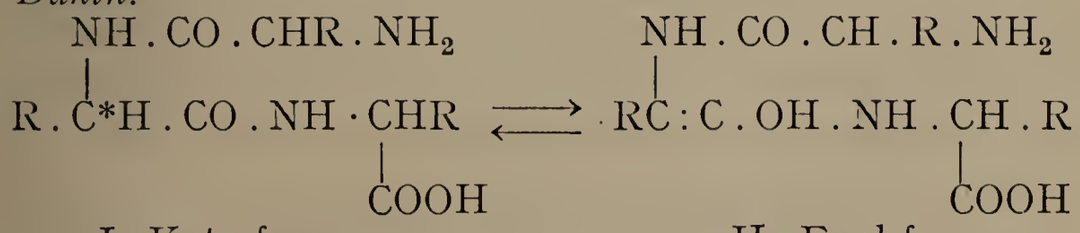
$$NH_2 . CH_2 . CO . NH . CH_2 . CO . NH_2 COOH \rightleftharpoons$$

I. Lactam.

$$NH_2 . CH_2C(OH) : N . CH_2C(OH) : N . CH_2COOH$$

II. Lactim.

Dakin.



I. Keto form. II. Enol form.

* *Zeitsch. physiol. Chem.*, 1909, **59**, 492 ; 1909, **60**, 311 ; 1910, **68**, 165.
† *Am. Chem. J.*, 1910, **44**, 48 ; *J. Biol. Chem.*, 1912, **19**, 357.

Actual experiments on the combination with alkali of compounds with the Dakin tautomerism have not yet appeared.

The difference in optical rotation between proteins and their salts with alkali observed by Pauli and M. Samec,* in direct analogy with the optical activity of the acid salts of the proteins already discussed, must not be confused with this variation in rotation with time.

It has already been mentioned that salts of the proteins with bases show a regular behaviour in their combination with alkali in increasing concentrations; the alteration in viscosity due to hydration of the alkali salt also varies in the same manner. Further, the order for different bases corresponds to that of their dissociation constants. With addition of alkali, the optical rotation is also observed to increase to a maximum corresponding to the saturation point. This value, however, *remains constant* on further addition of alkali. As in the case of acid, excess of alkali gives no decrease in rotation which can be attributed to repression of the ionisation. Thus, here again it is only the formation of the alkali protein and not the ionisation which affects the rotation.

Table 66.

Optical Rotation α° on addition of Alkalis.

	0·0 N.	0·005 N.	0·01 N.	0·02 N.	0·03 N.	0·05 N.
Sodium hydroxide .	1·02°	1·05°	1·13°	1·41°	1·52°	1·52°
Ammonia . . .	1·02°	1·04°	1·05°	1·06°	1·07°	1·07°

The optical rotation is thus affected in the same way by combination with both acid and alkali, and it is to be presumed that the effect on the albumin molecule is similar in both cases. Possibly the entrance of acid or metal affects the asymmetric carbon atom nearest the peptide linkage in the same way in the two cases.

* *Biochem. Zeitsch.*, 1914, **59**, 470.

CHAPTER IX

SALTS OF THE GLOBULINS: MIGRATION VELOCITY OF THE PROTEIN IONS

CLOSELY connected with the work on the caseinates are the researches of W. B. Hardy * on the salts of the globulins with acids and with alkalis. This fundamental work is distinguished by its modern point of view, and, on the chemical side, accurate conceptions are brought to bear on the solution of the problem. The globulin precipitate, obtained by slightly acidifying ox blood serum diluted ten times with water, was carefully washed for use in these experiments.

Various acids and bases dissolve globulin to a differing extent, and this property proved of great interest. A nephelometric method of determination was used, a known opacity being taken as the standard, for it was found that the solutions suffered a gradual change in opacity.

If the quantity in gram equivalents of hydrochloric acid required to dissolve 1 gm. of dry globulin is taken as unity, the values for various acids are those shown in the following table:—

Table 67.

HCl	1·0	H_2SO_4	1·91
HNO_3	0·995	Tartaric acid	1·994
$CHCl_2COOH$	1·0	Oxalic acid	1·9
CCl_3COOH	1·0	—	—
$CH_2ClCOOH$	1·05	Citric acid	3·0
H.COOH	1·25	H_3PO_4	2·9
CH_3COOH	5·7	H_3BO_3	Large
CH_3CH_2COOH	7·56	—	excess.

The strong monobasic acids, up to monochloracetic acid in the table, show the same power of dissolving globulin. Weaker

* "Colloidal solution: The Globulins," *Jour. Physiol.*, 1905, **33**, 251.

acids dissolve less of the protein, a measure of the strength being given by the value of $1/n$, where n is the number of gram equivalents of the acid necessary for the solution of unit weight of globulin. Strong and moderately strong dibasic acids have one-half, some of the tribasic acids one-third, of the capacity for solution of the globulin shown by monobasic acids. These results can be expressed alternatively by the statements that in formation of soluble globulin salts, the globulin and acid react in molecular proportions, or that the neutral globulin salts of polybasic acids are soluble in water, but not the basic salts.

Hardy determined in a similar way the extent to which globulin is dissolved by bases, with the following results, expressed as before :

Table 68.

KOH	1·00
NaOH	1·00
NH_4OH . . .	0·98
$Ba(OH)_2$ . . .	2·008

The solubility of the globulin in the diacid baryta is thus half as great as in the alkali hydroxide solutions. It is therefore true in this case also that the reaction which produces soluble salts with alkalis proceeds in molecular proportions. The neutral salt with baryta, but not the acid salt, is water-soluble. This result is very remarkable, and Hardy found that it also obtained for acid and alkali albumin prepared from egg albumin and dialysed free from salts.

The behaviour of globulin with very weak acids is simply explained on the basis of the considerable hydrolysis of the salt formed. This is shown by the precipitate of globulin, which only clears when an excess of the acid (for example, for acetic acid five times as much) is present. It then gives a complete solution as do the stronger acids. A comparison of the behaviour of globulin towards acetic acid with that towards ammonia is instructive, for the strength of the acid and base are much the same, the affinity constant being $1{\cdot}8 \times 10^{-5}$. Whereas acetic acid only dissolves a fifth as much as a strong monobasic acid, ammonia dissolves the same quantity as

potassium hydroxide, so that ammonium globulinate does not suffer an appreciable hydrolytic dissociation. This stronger combination with alkalis as compared with acids reflects the very acidic character of globulin.

The quantity of alkali hydroxide required to dissolve 1 gm. of globulin is of the order of 0·1 millimol, and this gives the high equivalent weight of 10,000 for these salts of the globulin. Double this quantity of alkali is necessary to neutralise the globulin when phenol phthalein is used as indicator, thus giving an equivalent weight of 5,000. The globulinate prepared in this way, when its conductivity is determined, behaves as if the globulin ions were divalent, thus making the molecular weight 10,000. Hardy, using Sackur's somewhat dubious method of calculation, obtains the value 5 for the valency, giving the altogether too high value of 25,000.

The conductivity of strong acids and bases, which is high owing to the great mobility of the H· and OH′ ions, falls off when neutralisation takes place. Thus the ratio of the conductivity of the salt and of the equivalent concentration of free acid or alkali is less than unity. On the other hand, it can become equal to 1, or even greater, if the salts of weak acids or bases are compared with the original acid or alkali.

Ammonium globulinate conducts the current much better than an equivalent solution of ammonium hydroxide; the ratio of the conductivities is 2·3. On the other hand the corresponding quotient for sodium globulinate and sodium hydroxide is 0·9.

In the results obtained by Hardy for the conductivity of globulin when acid or alkali is added, the measurements were made at such great dilutions that they are not unexceptionable either with regard to the large water-error or to the disturbing effects of hydrolysis. They lead, therefore, to values which are contradictory, and in particular far too high for the conductivity of the globulinate ion. One can, however, deduce the mobility of the globulinate ion, $v_{\text{Glob.}}$ from some of Hardy's experiments at a moderate dilution in the following way. The molecular conductivity μ at a dilution of V litres at 18° is given by the table on p. 124.

V.	μ Na Glob.	μ' NH_4 Glob.	α.	$v_{Glob.}$
135	35·2	48·9	0·685	7·55
251	38·4	53·4	0·75	7·45

Hence, on the widely accepted assumption * that the degree of dissociation of equivalent solutions of alkali globulin is the same :

$$\mu = \alpha\,(u_{Na} + v_{Glob.}) \text{ and } \mu' = \alpha\,(u_{NH_4} + v_{Glob.})$$

and therefore :

$$\alpha = \frac{\mu' - \mu}{u_{NH_4} - u_{Na}}.$$

At 18°, $\mu_{NH_4} - \mu_{Na} = 20$, and from this the values of α and $v_{Glob.}$ given in the table are deduced. A value for the absolute mobility of the negative globulin ion of at least $7{\cdot}77 \times 10^{-5}$ cm./sec. is thus obtained.

Hardy has determined the mobility of the globulin ion directly, using Whethan's method, in which the displacement of the bounding surface between the liquid under investigation and another of equal conductivity lying above it, on applying a known difference of potential is measured. The value of $9-11{\cdot}5 \times 10^{-5}$ cm./sec. was found in solutions of globulin chloride, and for sodium globulinate $7{\cdot}66 \times 10^{-5}$ cm./sec. for the mobility of the globulin ions. The latter value for the negative globulin ion is in excellent agreement with that which we have obtained from the conductivity. Acid globulin, particularly that formed with weaker acids, permits of the variation of the degree of dispersion of the globulin solution by addition of the acid in stages. On account of the hydrolysis which occurs, the size of the disperse particles can be varied over the wide range between a slight opacity and a thick precipitate. Hardy's work on globulin acetate shows that the electrophoretic behaviour is independent of the size of the globulin particles, which agrees with observations made on all kinds of colloids. It is well known that colloidal solutions as a rule contain disperse particles of varied size, as the production of colloids

* Discussed in the section on alkali caseinates, pp. 101—109.

composed of uniform particles necessitates special methods (Zsigmondy, Odèn). A separation of the particles according to size, when an electric field is applied, is not found to occur, because they all move with the same velocity in such circumstances. Now, according to Helmholtz's theory, equal mobility is due to equal density of charge on the surface; that is, the number of charges on one particle increases in proportion to the surface of the particle. Thus in the large complexes of the globulin precipitate, a considerable polyvalent effect occurs by each of the surface particles of the aggregates ionising as if it were a free globulin ion. But the charged particles united into aggregates, in spite of the unchanged masses of globulin produced by hydrolysis and carried along therewith, have actually a smaller total surface than the same number of globulin ions. Hence such aggregates will transport an equal quantity of electricity with less difficulty and more rapidly than the free ions. In this way the remarkably high mobility found by Hardy in very turbid globulin acetate can be explained.

A similar effect which also bears on the question is the increase in mobility of ions of the same size when the valency is increased. We give here two examples *; the analogous results for albumin will be added later:

		v. 18°.			v. 18°.
I.	H_2PO_4'	26·3	II.	$H_2P_2O_7''$	41·6
	HPO_4''	53·4		HP_2O_7'''	59·7
	PO_4'''	69·0		P_2O_7''''	81·4

If the charge on an ion is doubled while its surface remains constant, the transport of the same quantity of electricity occurs with doubled velocity. This actually happens, for example, in the ions of pyrophosphoric acid shown in series II. above. On the other hand, R. Wegscheider found that with organic ions of the same number of atoms, the ratio of the mobilities of di- and monovalent ions was not 2 but 1·8. Here the effect is not merely due to the change, but also to the alteration of hydration, an influence which is even more important in the case of complex protein ions. The large number of atoms in the molecule, and the heavy hydration have a retarding effect on

* Abbot and Bray, *J. Amer. Chem. Soc.*, 1909, **31**, 729.

the increase in mobility which the valency increase would produce.

Moreover, the formation of aggregates composed of both albumin ions and albumin molecules occurs, the latter produced by hydrolysis. In such complex ions (Hardy's pseudo-ions) the mobility will be determined by the proportions by weight and distribution of the component parts, and by other factors. We have already seen that such large ions, together with the metal or acid ions attached to them, will not pass through membranes, and consequently produce a high pressure in the osmometer.

The free diffusion of such ions, which has not, so far, been investigated, presents points of considerable interest. According to Nernst's theory, the following relation holds between the diffusion constant and mobility of ions :

$$D = \frac{2uv}{u + v} \cdot RT,$$

but in the case under consideration the high valency of the aggregates makes it necessary to test Hevesy's expanded formula.

A knowledge of the *mobility of the protein ions* is important, not only for the general chemistry of the proteins, but also for the solution of many biological problems. A supplementary exposition will therefore be devoted to it. In general, the proteins react with acids and bases with variable valency, and hence the number of possible protein ions is also variable. The simplest case is when, as in sodium caseinate, or in the globulinates, a maximum quantity of protein is taken up by a given quantity of alkali with formation of a definite neutral salt, giving only one ion of definite and not very high valency.

With increased addition of acid or alkali, however, new basic or acid valencies of the protein molecule are activated, with formation of ions of different valency ; and only after the maximum combination with acid or alkali can the existence of simple positive or negative valencies be again assumed. Under such conditions the maximum valency is exhibited. In this case the mobility of the protein ions also reaches its highest value. Between these points of saturation only the mean value of the

mobility can be obtained, and the mean valency of the protein ions deduced therefrom scarcely has a physical meaning.

The mobility of the protein ions can be obtained from measurements of conductivity in dilute solutions, provided a well-defined and well-dissociated protein salt, which gives one kind of negative ion only, is employed. The method of Whetham, using the moving bounding surface, is also applicable in suitable cases (Hardy). Yet another method has been adopted

Table 69.

1·256 *per cent. Horse-serum albumin, of original conductivity* $6{\cdot}77 \times 10^{-5}$. (*Temperature* 18°.)

Acid content.	C_H.	C_{Cl}.	λ corr.	Normality of protein ions.	Mobility of protein ions.
0·0075 N	$0{\cdot}238 \times 10^{-3}$	$5{\cdot}386 \times 10^{-3}$	$4{\cdot}489 \times 10^{-4}$	$5{\cdot}148 \times 10^{-3}$	11·11
0·008	$0{\cdot}240 \times 10^{-3}$	$6{\cdot}071 \times 10^{-3}$	$5{\cdot}494 \times 10^{-4}$	$5{\cdot}831 \times 10^{-3}$	13·08
0·01	$0{\cdot}427 \times 10^{-3}$	$6{\cdot}887 \times 10^{-3}$	$7{\cdot}152 \times 10^{-4}$	$6{\cdot}460 \times 10^{-3}$	20·08
0·012	$0{\cdot}668 \times 10^{-3}$	$8{\cdot}367 \times 10^{-3}$	$9{\cdot}275 \times 10^{-4}$	$7{\cdot}699 \times 10^{-3}$	21·95
0·014	$1{\cdot}082 \times 10^{-3}$	$9{\cdot}502 \times 10^{-3}$	$11{\cdot}761 \times 10^{-4}$	$8{\cdot}420 \times 10^{-3}$	25·29
0·015	$1{\cdot}292 \times 10^{-3}$	$9{\cdot}873 \times 10^{-3}$	$12{\cdot}818 \times 10^{-4}$	$8{\cdot}581 \times 10^{-3}$	26·58
0·016	$1{\cdot}392 \times 10^{-3}$	$11{\cdot}333 \times 10^{-3}$	$14{\cdot}614 \times 10^{-4}$	$9{\cdot}939 \times 10^{-3}$	28·25
0·02	$3{\cdot}043 \times 10^{-3}$	$14{\cdot}786 \times 10^{-3}$	$22{\cdot}909 \times 10^{-4}$	$11{\cdot}443 \times 10^{-3}$	30·98

by Pauli and Sven Odèn,* whereby the mobility can be directly found in cases in which the concentrations of each of the ions can be accurately determined, *e.g.*, in the case of the hydrochlorides of the proteins. If C_H and C_{Cl} are the concentrations of $H^{\cdot}$ and Cl' ions as determined electrometrically, then the fraction of the Cl' ion content due to the protein, or the normality of the protein ions is $C_{Cl} - C_H$. If the mobilities of the $H^{\cdot}$, Cl' and protein ions are u_H, v_{Cl}, and u_x, then the conductivity of the mixture of acid and protein, λ, is the sum of the products of the concentrations and the mobilities of the respective ions :

$$\lambda = C_H u_H + C_{Cl} v_{Cl} + (C_{Cl} - C_H)\, u_x,$$

and hence the mobility of the protein ions

$$u_x = \frac{\lambda - (C_H u_H + C_{Cl} v_{Cl})}{C_{Cl} - C_H}.$$

* *Anzeiger. Akad. Wissensch. Wien.*, 1913, No. 24.

Results for serum albumin are given in Table 49. The limiting value as extrapolated from the curve of protein-normality and ion mobility (Fig. 27) is about 33.

The rise of the mobility of the protein ions to a maximum corresponds to the increase in valency of the ions, which, as we have seen above in the case of inorganic ions, causes a proportional increase in the migration velocity when the size of the ions remains the same.

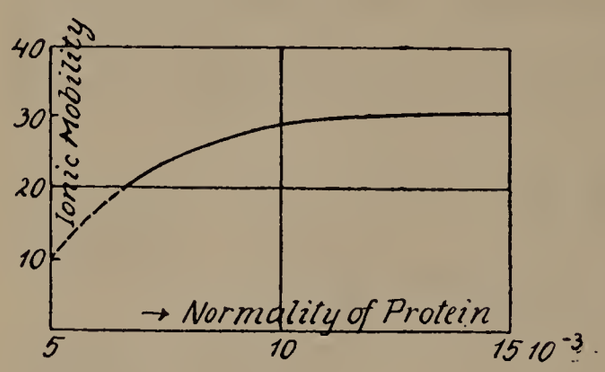


FIG. 27.

Addition of alkali causes a gradual decrease in the charge on the protein ions with a falling off in migration velocity and finally a reversal with increasing mobility in the opposite direction. A similar effect with inorganic colloids, to which we can ascribe, as Zsigmondy has done, the multiple charge of a single ion, has been described by Burton.* He measured the rate of electrophoresis of gold and silver suspensions before and after addition of increasing quantities of aluminium sulphate, of which the trivalent metallic ion is the source of the positive charge. The results of some of these experiments are given below, the sign of the electric charge being stated as well as the absolute migration velocity.

Table 70.

Colloidal Silver treated with Aluminium Sulphate.

Millimols $\frac{Al_2(SO_4)_3}{2}$ per litre.	V. cm/sec. at 18°.
0	$-22{\cdot}4 \times 10^{-5}$
0·0052	$-7{\cdot}2 \times 10^{-5}$
0·0140	$+5{\cdot}9 \times 10^{-5}$
0·0284	$+13{\cdot}8 \times 10^{-5}$

A comparison of the behaviour of a protein and a colloidal metal is useful in spite of the apparent difference of the two

* *Phil. Mag.*, 1906 (6), **12**, 472.

systems in the origin of the electric charge—the former appears as a polyvalent base or acid, the latter as a Nernst electrode, sending out metal ions. There is a remarkable agreement between the chemical and the electro-chemical conceptions of the mechanism. We may assume, nevertheless, that the charge of a colloidal metal particle may correspond to the distribution on a charged conductor, whereas that on a protein molecule is probably localised at the discrete points where the acid or basic valencies occur.

It is well known that potential differences occur at the boundary between two solutions of electrolytes when they contain ions of differing velocity. According to Nernst, the potential difference is produced by the more mobile ion, *e.g.*, the $H^{\cdot}$ ion of a dilute hydrochloric acid solution advancing more rapidly than the Cl' ion, and thus conferring its charge on the adjoining second electrolyte. When, for example, dilute equivalent solutions of HCl and NaCl are in contact, the so-called diffusions potential at the interface is due only to the different mobility of the $H^{\cdot}$ and $Na^{\cdot}$ ions. For this simplified case, in which binary electrolytes giving two univalent ions in equivalent concentration are considered, Nernst's theory gives the following equation :

$$E = \frac{RT}{F} \log_e \frac{u_1 + v_1}{u_2 + v_1},$$

in which E is the electromotive force, R the gas constant, T the absolute temperature, F the quantity of electricity carried by a univalent ion, u_1 and u_2 the mobilities of the cations, and v_1 the mobility of the common anion. A case as simple as this is difficult to realise in the salts of the albumins, as their dissociation is less than that of most salts, and would have to be determined accurately in each individual case. A further condition which deserves notice is as follows. According to all experience, the proteins yield salts with protein ions of increasing valency when increasing quantities of alkali or acid are added. A uniform constitution in the protein salt is to be expected in low concentrations of acid or of alkali when the soluble protein salt shows the protein ion of lowest valency. Again when the

protein is saturated in presence of excess of acid or of alkali only the highest possible valency of the protein ion comes into consideration. In all intermediate stages the protein salts with acids or bases give mixtures of ions of differing valency and mobility. This is why the mobilities of the protein ions, as determined by Pauli and Odèn for increasing addition of acid, lie on a *continuously* rising curve.

Henderson * has deduced a general formula on the basis of Nernst's theory, for the diffusion potential which applies to any ions or mixtures. Thus—

$$E = \frac{RT}{F} \cdot \frac{(U_1 - V_1) - (U_2 - V_2)}{(U'_1 + V'_1) - (U'_2 - V'_2)} \cdot \log_e \frac{U'_1 + V'_1}{U'_2 + V_2}$$

in which

$$\begin{aligned} U_1 &= u_1C_1 + u_2C_2 + \ldots\ldots \\ V_1 &= v_1C_1 + v_2C_2 + \ldots\ldots \\ U'_1 &= u_1w_1C_1 + u_2w_2C_2 + \ldots. \\ V'_1 &= v_1w_1C_1 + v_2w_2C_2 + \ldots. \end{aligned}$$

When $u_1, u_2 \ldots.., v_1, v_2 \ldots.$ are the mobilities, $w_1, w_2 \ldots.., w_1, w_2 \ldots.,$ are the valencies, and $C_1, C_2 \ldots., C_1, C_2 \ldots$ are the concentrations of cations and anions respectively.

Pauli and Matula have carried out experiments (not yet published) with a view to determining the mobility of the protein ions in this way. Various circuits, in which albumin salts were included, were built up, the following being a typical example. Solutions of the protein salt and of hydrochloric acid were included between two normal calomel electrodes. In the second circuit given below, concentrated potassium chloride solution, which gives the equally mobile $K^{\cdot}$ and Cl' ions, was interposed to eliminate the diffusion potential.

Circuit I.

Hg/HgCl . N KCl/0·002 N HCl + 1 per cent. albumin/
0·002 N HCl/N KCl . HgCl/Hg.

* *Zeitsch. physikal. Chem.*, 1907, **59**, 118; 1908, **63**, 325.

Circuit II.

Hg/HgCl . N KCl/0·002 N HCl + 1 per cent. alb./KCl conc./
0·002 N HCl/N KCl . HgCl/Hg.

$T = 293°$
$E_{I.} = 0{\cdot}025$ volt.
$E_{II.} = 0{\cdot}059$ volt.
Diffusion potential $= E_{II.} - E_{I.} = 0{\cdot}0322 \pm 0{\cdot}0005$ volt.

A careful analysis showed that the albumin solution contained ammonium sulphate in a concentration of 0·0046 N, owing to the purification of the albumin by the precipitation method. Hence $C_{\frac{SO_4}{2}} = C_{NH_4} = 46 \times 10^{-5}$. The hydrion concentration in the albumin—HCl mixture was $5{\cdot}4 \times 10^{-5}$ N, and the concentration of chlorine ions 170×10^{-5} N. In the solution of pure hydrochloric acid $C_H = C_{Cl} = 170 \times 10^{-5}$ N. Thus combination of hydrion with the albumin accounts for the difference, 164×10^{-5} N, in the hydrion concentration; and this is equal to the normality of the albumin salt. Also,

$$u_H = 330,\ v_{Cl} = 68,\ v_{SO_4} = 71,\ u_{NH_4} = 67.$$

These various values were then substituted in Henderson's equation, which was then solved for U_{Alb}. The value obtained is 5—8, if it is assumed that the albumin ion is monovalent in this low concentration of acid. This result, as far as order of magnitude is concerned, is in satisfactory agreement with that obtained by Pauli and Odèn for the mobility in low concentrations of acid. The determination of migration velocity by the method of diffusion potentials has proved in practice to be a somewhat complicated matter and not at all sensitive; but such methods have a special importance in elucidating the origin of the bio-electric current.

The behaviour of *salts of the proteins on electrolysis* is closely connected with the valency of the protein ions. As we now know that proteins form typical metallic salts with alkalis, we should expect that when alkali proteins are electrolysed the metallic ion would appear at the cathode, and the protein ion at the anode. The former ions react with the water with formation of the original alkali, the latter in the case of a protein

is insoluble, and is precipitated as such. The alkali caseinate described above, as it is capable of more accurate definition, is particularly suitable for investigations of this kind, as in solution the concentration of free hydroxyl ions and of alkali ions is so small as not to come into consideration during electrolysis.

If a second electrolytic cell in which the products can be easily estimated, *e.g.*, a silver voltameter, is included in the main circuit with the solution under investigation, the ratio of the weight of silver deposited to the quantity of protein precipitated can be determined. The equivalent of the latter can then be deduced from Faraday's law. Thus in the case of the two caseinates

I. $K_3 . (\text{Caseinate})'''$ and
II. $K_3 . ([\text{Caseinate}]''' . \text{Casein})$

when introduced into such a circuit in separate cells, the same current will precipitate at the anode twice as much casein in solution II. as in solution I.

As it happens, T. B. Robertson,* *inter alia*, has made a number of measurements of the electro-chemical equivalents in such mixtures of casein and alkali. Solutions containing 50×10^{-5} gm. equivalents (0·5 millimol) and 100×10^{-5} gm. equivalents (1 millimol) of potassium hydroxide per gram of casein respectively correspond accurately to the caseinates mentioned above.

Robertson's measurements of the electro-chemical equivalent of a protein are, so far, the only ones of their kind, and it is to be regretted that he is so much under the influence of his theory of the formation of protein ions that he has failed to make the deductions which would be warranted by the importance and accuracy of his work.

His apparatus comprised a U-tube with platinum wires leading into it. The two limbs of the U-tube could be connected by a three-way stop-cock, or could be separately emptied. The anode was a spiral of platinum wire of such a length as to permit of a suitable current density, and at the same time retain

* "Physical Chemistry of the Proteins," p. 176, where the literature is quoted.

the adhering precipitate of casein. The concentration of casein in the solutions at the electrodes was found by determinations of the alteration in refractive index with a refractometer. A silver voltameter arranged for titrimetric estimation was included in the circuit. The work was carried out at a temperature of 30°. The liquid at the anode always became poorer in casein but unchanged in reaction towards indicators, whereas that at the cathode also became weaker in casein, but developed a strongly alkaline reaction. This behaviour is easy to understand from the conception of the structure of the caseinates which we have emphasised in the foregoing pages.

When, on the other hand, we apply Robertson's conception, difficulties arise at once with these results. He maintains that the alkali enters the molecule at the peptide linkage, with production of two oppositely charged protein ions :

$$\begin{array}{c} H \\ | \\ R\,.\,N'' \\ | \\ OH \end{array} \text{ and } KO\,.\,C\,.\,R^{++}$$

The former would move to the anode, the latter towards the cathode. Robertson further assumes that the negative ion reacts with water at the anode with liberation of oxygen and formation of solid casein, and that the positive ion reacts with water at the cathode, hydrogen being liberated and casein formed. The casein at this pole dissolves at once in the potassium hydroxide which is produced at the same time. Thus he quite overlooks the fact that these protein products do not give the original casein when they react with water, but can only give a new protein product, which must be different at the two electrodes, the new substances being fractional parts of the casein. According to Robertson the secondary reaction at the cathode must always lead to the saturation of the base with protein, and thus even with varying proportion of alkali to casein in the original solution, a *constant electro-chemical equivalent* would be found for various caseinates. The observed values, however, show no such constancy, for with increasing content of alkali the electro-chemical equivalent decreases.

This variation was corrected for by Robertson on the basis of the following line of reasoning. If the anode with the adhering precipitate of casein is immersed in the original caseinate solution, part of the precipitate passes into solution ; this is particularly the case in the solutions containing more alkali and less casein. This quantity is determined, taken as a loss during

Table 71.

50×10^{-5} *equivalents* KOH *per gram of casein.*

Per cent. casein.	Current in ampères.	Time of electrolysis.	Gram casein lost from the solution.	Electro-chemical equivalent, milligram per coulomb.
6	$11{\cdot}51 \times 10^{-4}$	2 hr. 15′	0·2155 ± 0·0175	23·1 ± 1·9
4	$9{\cdot}47 \times 10^{-4}$	2 hr. 0′	0·1645	24·1 ± 2·6
4	$18{\cdot}05 \times 10^{-4}$	2 hr. 15′	0·3815	26·1 ± 1·2
3	$10{\cdot}54 \times 10^{-4}$	2 hr. 0′	0·1810	23·8 ± 2·3
3	$17{\cdot}36 \times 10^{-4}$	2 hr. 0′	0·3125	25·0 ± 1·4
2	$11{\cdot}22 \times 10^{-4}$	2 hr. 0′	0·1810	22·4 ± 2·2
2	$15{\cdot}84 \times 10^{-4}$	1 hr. 25′	0·1678	20·8 ± 2·2
			Mean :	23·6 ± 2·7

Table 72.

100×10^{-5} *equivalents* KOH *per gram of casein.*

Per cent. casein.	Current in ampères.	Time of electrolysis.	Gram casein lost from the solution.	Electro-chemical equivalent, milligram per coulomb.
3	$12{\cdot}03 \times 10^{-4}$	2 hr.	0·0988 ± 0·0175	11·4 ± 2
2	$12{\cdot}56 \times 10^{-4}$	1 hr.	0·0493 ± 0·0175	10·9 ± 3·9
			Mean :	11·15 ± 3

electrolysis, and the weight of the precipitate corrected by this factor. This argument is, however, erroneous, for the observations on the solution of the precipitate are not made under the conditions which prevail in the neighbourhood of the anode. In that area, in consequence of the more rapid transference of the K· ions (Hittorf), the liquid rapidly becomes weak in caseinate and thus the quantity of the very substance to which the

capacity of dissolving the casein may be attributed is diminished. Apart from the fact that Robertson's corrected values still show clearly a tendency to decrease with increasing concentration of alkali, no safe basis for the correction applied by him exists at all.

We give, therefore, the uncorrected, actual experimental results of this author in the tables on p. 134.

A glance at these results shows that, as a matter of fact, salt I. (Table 72) exhibits half the electro-chemical equivalent of salt II. (Table 71), as we should expect from the constitution of the caseinates. For the complex ion [casein (caseinate''')] the mean value of 23·7 mg. is obtained; for the simple ion (caseinate)''' the value of 11·15 mg. per coulomb. If the equivalent weight be calculated therefrom, taking 96,540 as the number of coulombs required to transport 1 gm. equivalent of an ion, we obtain 1,076 as the equivalent weight of casein which has been taken to be about 1,000 in the preceding pages.

These electro-chemical measurements, therefore, provide valuable evidence in favour of the constitution of the caseinates which we have previously assumed.

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